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Final Thesis for the Achievement of the Engineering Degree in Marine Science and the Acquisition of the Startup Patent Diploma

Speciality: Marine Biotechnology

Chitin, Chitosan production using marine waste sources Characteristics and applications

Presented by: BOUCHACHIA Meriem

Supported on 12/ 07/2023, in front of the jury composed of:

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بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

فَٱذۡكُرُونِيٓ أَذۡكُرۡكُمۡ وَٱشۡكُرُواْ لِي وَلَا تَكۡفُرُونِ ١٥٢ [سورة البقرة]

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With boundless love and heartfelt gratitude,

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Abstract

This research focuses on the extraction, characterization, and application of chitin and chitosan from marine waste sources. Chitin and chitosan, derived from abundant marine waste, possess remarkable properties that make them highly desirable in various industries.

The importance of this research is twofold. Firstly, the lack of chitosan products in the Algerian market presents a significant gap in the industry. By extracting chitosan from marine waste, this research contributes to meeting market demands and opens avenues for economic growth and product diversification. Secondly, the utilization of waste materials is a pressing concern worldwide. Marine waste, if not properly managed, poses environmental hazards. By extracting chitin and chitosan from these waste sources, this research tackles the waste management issue while simultaneously producing valuable compounds with versatile applications.

The primary objective of this research is to develop and optimized an extraction approach for chitin and chitosan. This approach aims to streamline the extraction process by reducing processing time, minimizing reagent consumption, and optimizing yields. By achieving these objectives, the research aims to promote sustainable waste utilization, meet market demands, and demonstrate the broad potential of chitosan in various industries.

The research methodology involves implementing a chemical extraction process inspired by multiple protocols. By combining and optimizing various extraction techniques, the study aims to enhance the efficiency and effectiveness of chitin and chitosan extraction from marine waste sources. The resulting chitosan will be meticulously tested and characterized to ensure its quality, purity, and compatibility with different applications.

The research findings affirm our hypothesis that chitosan derived from marine waste can be effectively extracted and utilized in various industries. This addresses the scarcity of chitosan products in the Algerian market, opening up opportunities for economic growth and technological advancement. The demonstrated antibacterial properties against bacteria and fungi, wound healing capabilities, moisturizing effects, and bio stimulant potential of chitosan validate its commercial viability and highlight its significance as a valuable resource derived from waste materials.

In conclusion, this research contributes to bridging the gap in the Algerian market by developing an efficient extraction method for chitin and chitosan from marine waste. The utilization of waste materials through chitosan extraction not only addresses environmental concerns but also provides new economic prospects. The diverse applications and commercial viability of chitosan emphasize its potential as a valuable resource, supporting sustainable waste management practices and promoting economic growth.

Keywords :

1. Chitosan
2. Characterization
3. Marine waste utilization
4. Applications
5. Extraction
6. Algerian market

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Introduction

Algeria, a coastal country with a vast stretch of the Mediterranean Sea, possesses abundant marine resources that hold tremendous potential for economic and environmental value. The coastal regions of Algeria are home to diverse marine ecosystems, rich in biodiversity and teeming with marine life. However, along with the natural beauty and ecological significance of these coastal areas, there exists a pressing concern regarding the management of marine waste. The accumulation of marine waste poses significant environmental challenges, impacting not only the delicate marine ecosystems but also the coastal communities and their socio-economic activities.

Recent advancements in marine biotechnology have shed light on the potential of marine waste as a valuable resource for the extraction of bioactive compounds. One such resource of immense interest is chitin and chitosan, derived from the exoskeletons of crustaceans and marine organisms. Chitin and chitosan have versatile properties and find wide-ranging applications in various fields, including medicine, agriculture, cosmetics, and industry.

This dissertation aims to explore the extraction, characterization, and application of chitin and chitosan obtained from marine waste sources. By investigating different extraction methods and assessing their efficacy, as well as evaluating the performance of chitosan in multiple fields.

To achieve this objective, the dissertation is divided into three main chapters:

The first chapter, “**Literature review”**, provides a comprehensive overview of chitin and chitosan, including their history, properties, characterizations, extraction methods, and diverse applications. This chapter serves as a foundation for understanding the fundamental aspects of chitin and chitosan, their structural characteristics, and the various approaches employed for their extraction from marine waste. Additionally, it explores the wide range of applications in fields such as pharmaceuticals, cosmetics, agriculture, and paper industry, highlighting the potential benefits and significance of utilizing chitin and chitosan in these areas.

The second chapter, “**Experimental section”**, focuses on the collection and treatment of raw materials, as well as the equipment used for the extraction process. Different extraction methods will be employed and compared to determine the most efficient approach for obtaining high-quality chitin and chitosan from shrimp shell waste. The extracted biomolecules, chitin and chitosan, will be thoroughly characterized through various analytical techniques, including physicochemical analysis. Furthermore, this chapter will explore the diverse applications of chitosan in fields such as pharmacy, cosmetics, agriculture, and paper industry. The evaluation of chitosan's performance in these applications will provide valuable insights into its potential benefits and effectiveness.

The third chapter, “**Results and discussion”**, presents the findings obtained from the experimental analysis. The results will be discussed in detail, addressing the efficiency of different extraction methods and the characteristics of chitin and chitosan derived from marine waste sources. Moreover, the chapter will delve into the application-specific results, including the performance of chitosan in wound healing, anti-inflammatory effects, antibacterial potential, and plant growth stimulation. The discussion will critically analyze the results, highlighting their significance and implications for marine biotechnology and sustainable resource utilization.

In the final section, a “**general conclusion”** will be drawn based on the results and discussions presented in the previous chapters. This conclusion will summarize the key findings, restate the research objectives, and provide insights into the overall contributions of the study

I. Literature review

I. Chitin

1. History

Over 200 years ago, the French botanist Henri Braconnot, made an important discovery while conducting research on edible mushrooms. He identified a new polysaccharide that he called (fongine/fungine) (**CRINI and LICHTFOUSE, 2019).**

This preceded the discovery of cellulose by about three decades. Chitin was later discovered in the cuticles of insects by Odier in 1823, and the name "Chitin" was derived from the Greek word meaning tunic, covering or envelop **(CASADIDIO *et al*., 2019).**

In the years following its discovery, chitin and its derivative chitosan, gained significant interest and became the focus of numerous patents. Chitin, the most abundant naturally occurring biopolymer with amino acid and sugar components, is second only to cellulose in terms of abundance among naturally occurring polymers, chitin's chemical composition comprises N-acetyl-D-glucosamine units that are linked together through β-(1-4) glycosidic bonds**. (AHMED and IKRAM ,2017).**

Figure 1:Henri Braconnot, 1780–1855 (crini and lichtfouse, 2019)

2. Structure

Chitin $(C_8H_{13}O_5)$n, is a white, hard, inelastic, nitrogenous polysaccharide, is a copolymer of N-acetyl-D-glucosamine and D-glucosamine units linked with β-(1-4) glycosidic bonds, where N-acetyl-D-glucosamine units are predominant in the polymeric chain. **(THOMAS *et al*., 2020).**

The structure of this nitrogenous polysaccharide is similar to that of cellulose, except for the replacement of the hydroxyl group at the carbon with an acetylamino group (-NHCOCH3) **(Figure 02).**

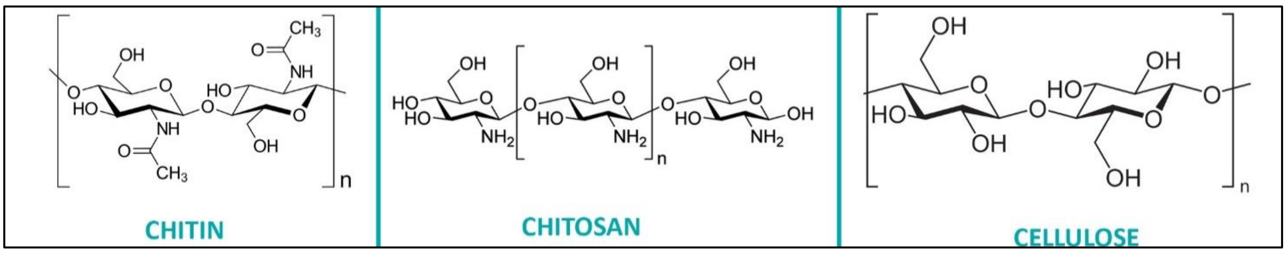


Figure2 : Chitin -Chitosan -Cellulose structure (CRINI and LICHTFOUSE, 2019)

Chitin is commonly represented as a linear long-chain homopolymer, composed of N-acetyl glucosamine units, with different polymorphic forms (α-, β-, and γ-chitin) as shown in the Figure 03, depending on the source, where their arrangement of macromolecular chains varies **(PELLIS *et al.*, 2022)**. Specifically, in α-chitin, the chains are arranged antiparallel to each other, while in β-chitin, they are parallel, and in γ-chitin, two parallel chains and one antiparallel chain form the structure **(CRINI and LICHTFOUSE ,2019).**

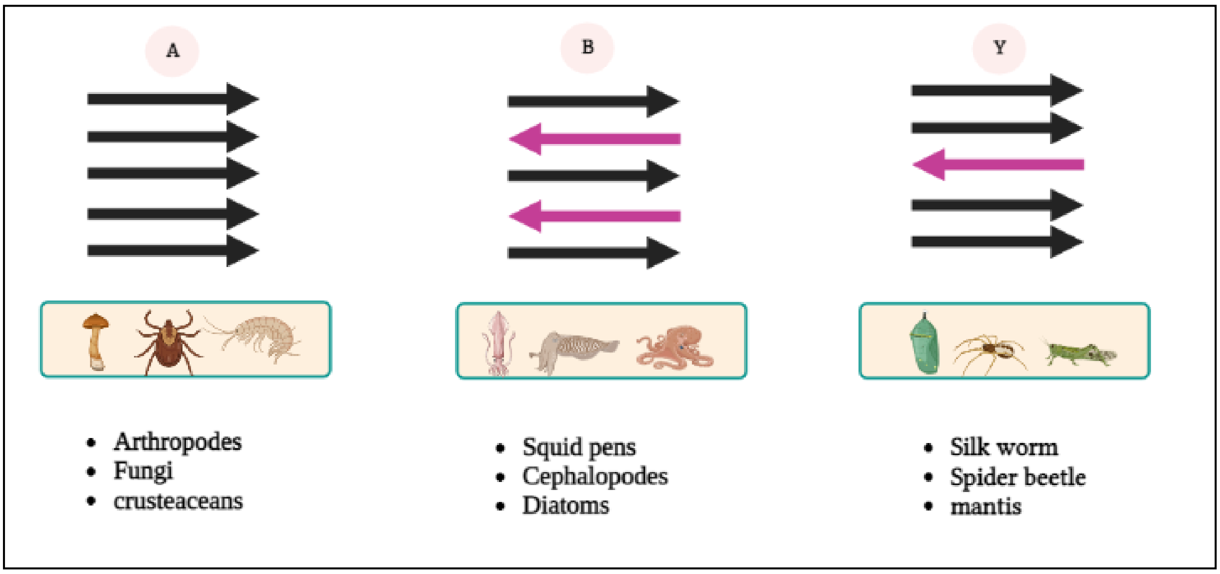


Figure 3:Chitin polymeric Forms (α-, β-, and γ-)

3. Sources of Chitin

Chitin is a naturally occurring polysaccharide that has a wide distribution in nature. It is found in various organisms such as arthropods, fungi, cephalopods, and some marine animals **(PELLIS *et al.*, 2022).**

In arthropods, chitin provides structural support and protection by forming a complex matrix with other proteins and polysaccharides. In fungi, it is a vital component of the cell wall, helping to maintain cell shape and integrity.

Additionally, chitin is present in some marine organisms, including krill, copepods, and certain fish species. Presently, the majority of chitin production is derived from crustaceans, such as shrimp, crab, and shellfish. Microbial sources are also used as a source of chitin. **(SANTOS *et al.*, 2020).**

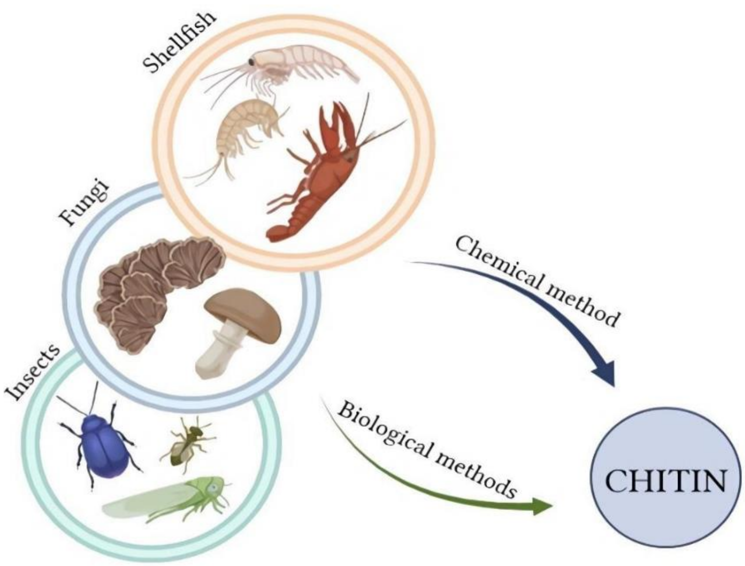


Figure 4:The sources of chitin for chitosan preparation (kulka and sionkowska, 2023

4. Chitin isolation

The cuticles of various marine crustaceans such as crabs, shrimps, and lobsters, as well as fish scales, are the major sources for the large-scale production of chitin and chitosan due to the abundance of low-cost by-products of seafood processing industry **(ZAINOL ABIDIN *et al*., 2020).** These sources contain chitin (15-40%), along with proteins (20-40%) and calcium carbonate (20-50%) as the two major components. Other minor components include pigments, lipids, and minerals like metals and salts (THOMAS *et al*., 2020)**.**

Crustacean shells contain a matrix of protein and chitin that is covalently bonded and extensively calcified to produce tough shells. Chitin can be extracted from these shells using either a chemical or a biological (enzymatic) process. However, the biological process requires a longer reaction time for deproteinization compared to the chemical process, making the chemical process more suitable for large-scale production of chitin. **(HASAN *et al*., 2022).**

Separating chitin from the shell involves the removal of the two major constituents, protein and minerals, through deproteinization and demineralization processes, respectively. Decolorization may also be carried out to remove pigments **(HOSNEY *et al*., 2022) ,**TheFigure below, provides a simplified representation of the chitin preparation process from aquatic crustacean biowaste.

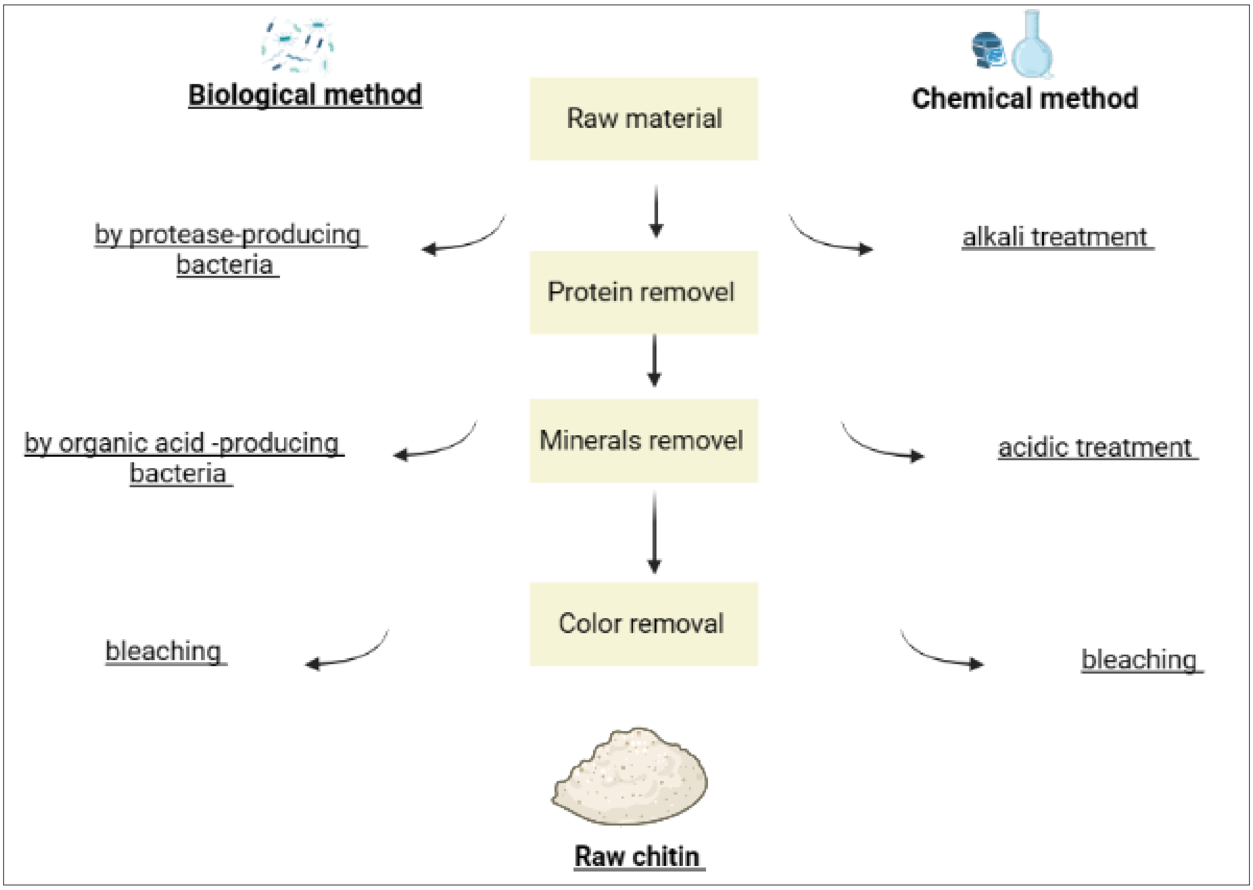


Figure 5:Chitin preparation process from aquatic crustacean biowastes

A- Chemical extraction

The basic steps for chitin isolation from crustacean shells are as follows:

- Demineralization (acid removal of calcium carbonate).
- Deproteinization (removal of proteins).
- Depigmentation (removal of pigment).

Demineralisation:

Demineralization of source material (crustacean shells) starts with the removal of minerals such as calcium carbonate ($CaCO_3$), The most common reagents are HCl, HNO_3, H_2SO_4, CH_3COOH, and HCOOH, generally HCl seems to be best one. Then the demineralized shell is filtered under vacuum and washed with distilled water. **(THOMAS *et al*,2020)**

The process of demineralization varies depending on various factors such as the type of shells, extraction time, temperature, size of the shell, concentration of acid, and the ratio of solute to solvent. However, it is important to note that a high concentration of acid, prolonged process time, and high temperature can have a negative impact on the properties of chitin. **(HASAN *et al*., 2022),(ZAINOL ABIDIN *et al*., 2020).**

In this process, calcium carbonate reacts with acid (HCL) and decomposes to form water-soluble calcium salt with the release of carbon dioxide (CO_2), as shown in the following equation **(HASAN et al., 2022):**

$$2HCl + CaCO_3 \rightarrow CaCl_2 + H_2O + CO_2 \uparrow .$$

Deproteinization:

Deproteinization is commonly carried out through an alkaline treatment, which leads to the disruption of the chemical bonds between protein and chitin. The major protein component, albumen, breaks down into water-soluble amino acids as a result. Various reagents such as NaOH, Na_2CO_3, $NaHCO_3$, KOH, K_2CO_3, Ca $(OH)_2$, Na_2SO_3, $NaHSO_3$, $CaHSO_3$, Na_3PO_4, and Na_2S can be used for this process. **(SANTOS *et al*., 2020).**

The extent of deproteinization required for chitin extraction can vary depending on the source of the crustaceans. Alkali treatment is often used to remove the protein from the shells, but this can also result in the partial deacetylation of chitin and a decrease in its molecular weight. **(THOMAS *et al*., 2020) (ZAINOL ABIDIN *et al*., 2020)**

Decolorization

Decolorization of chitin is the final stage of chitin preparation, the shells of crustaceans may contain lipids and pigments, which can form complexes with chitin. To remove these pigments, an extraction step is carried out using hydrocarbon solvents like acetone, chloroform, ethyl acetate, or ethanol. **(THOMAS *et al*,2020) (HASAN *et al*., 2022).**

B- Biological extraction

An alternative method for chitin extraction is the biological method, which involves the use of microorganisms to degrade the organic matter surrounding chitin.

Enzymatic demineralization:

The enzymatic demineralization of crustacean shells involves the dissolution of mineral and protein components by organic acids produced by microorganisms, specifically lactic acid-producing bacteria. This reaction occurs when the organic acids react with calcium carbonate, which is present in the shells, resulting in the precipitation of calcium salts. These salts are subsequently removed from the culture medium, and the organic salts are separated from the precipitated material through washing. The precipitated organic salts can then be utilized as preservatives and anti-icing agents. **(HASAN *et al*., 2022).**

Enzymatic deproteinization:

Enzymatic deproteinization is a process in which various proteases, including alcalase, pepsin, papain, pancreatin, devolvase, and trypsin, are utilized to remove proteins from raw materials. These proteolytic enzymes can be obtained from a variety of sources such as plants, microbes, and animals. By eliminating the need for preliminary processing steps, proteolytic enzymes reduce processing time and increase efficiency. **(AHMED and Ikram, 2017) (HASAN *et al*., 2022) (PELLIS *et al*., 2022).**

Fermentation:

Enzymatic chitin extraction can be expensive due to the high cost of enzymes. However, the cost can be reduced by using a fermentation process to perform deproteinization. Proteolytic enzymes produced by lactic acid bacteria can be used to hydrolyse proteins in the shells, and this process is activated by a low pH in the medium. This method has the advantage of allowing for the recovery of valuable by-products such as proteins, enzymes, and pigments that can be used in the food industry, thereby adding further value to the overall process. **(SANTOS *et al*., 2020).**

5. Properties of Chitin

Physicochemicalproperties:

Chitin, as a biopolymer, exists as a colorless, crystalline or amorphous powder, is a highly crystalline material similar to cellulose, and its solubility and reactivity are affected by the presence of specific functional groups such as -NH2 and -OH. Chitin consists of β-(1,4)-linked N-acetyl-D-glucosamine units, which provide the material with unique chemical and physical properties. **(CRINI and LICHTFOUSE, 2019).**

The presence of -NH2 and -OH groups in chitin makes it more hydrophilic than cellulose. These functional groups make chitin partially soluble in some polar solvents such as dilute acids, acetic acid, and certain ionic liquids. The solubility of chitin also depends on its degree of acetylation, with low levels of acetylation resulting in higher solubility in some solvents. **(THOMAS *et al*., 2020).**

The -NH2 and -OH groups in chitin also make it reactive towards various chemical modifications. For instance, chitin can undergo deacetylation to produce chitosan, a material with enhanced solubility and reactivity due to the presence of free amino groups. Additionally, the -NH2 and -OH groups in chitin can react with various chemical groups such as carboxylic acids and aldehydes to form derivatives with unique properties for various applications. **(AHMED and IKRAM, 2017).**

Overall, the unique chemical and physical properties of chitin, influenced by the -NH2 and -OH functional groups, make it a versatile material with potential applications in various fields, including biomedicine, food, agriculture, and environmental industries. **(THOMAS *et al*., 2020) (AHMED and IKRAM, 2017).**

Miscellaneous Properties

Chitin is a versatile polysaccharide that has several unique properties **(HASAN *et al*., 2022)**, some of them are mentioned in the figure below:

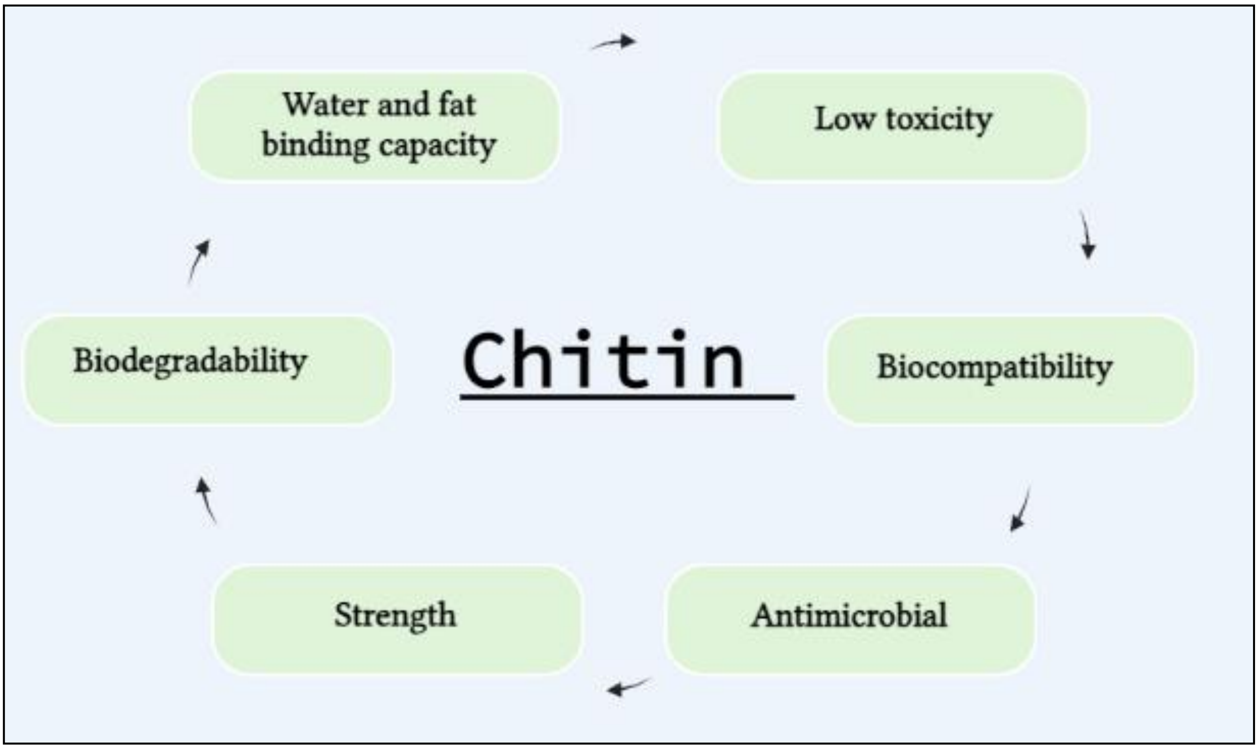


Figure 6:Chitin Proprties

II. Chitin main derivative: Chitosan

1. History

Chitosan has a history dating back to 1859, when C. Rouget treated chitin with strong alkali to create a new substance that could be dissolved in acidic aqueous solutions. This product was called "chitinemodifiée," or modified chitin **(CRINI and Lichtfouse, 2019)**.

In the 1930s, X-ray diffraction was used to characterize the structure of chitin and chitosan. In 1936, Rigby obtained two patents for producing chitosan from chitin and for making films and fibres from chitosan, which led to the first use of chitosan in the papermaking industry **(MUZZARELLI *et al*,2012)**.

Since the 1970s, many manufacturers have produced and marketed chitin and chitosan products, resulting in numerous patents and a wealth of scientific literature. Today, there are over 2000 applications of chitin, chitosan, and their derivatives in hypes of fields **(BEREZINA ,2016), (ANNU *et al*,2017), and (KARTHIK *et al* ,2017).**

Figure7 : Charles Marie Benjamin Rouget (CRINI and Lichtfouse, 2019)

2. Structure

Chitosan, which is the primary derivative of chitin, is a natural biopolymer that is obtained through N-deacetylation of chitin under alkaline conditions. This linear polysaccharide is composed of D-glucosamine units, which are 2-amino-2-deoxy-(1-4)-β-D-glucopyranose residues **(AHMED and IKRAM, 2017).**

There is a naming convention that distinguishes between chitin and chitosan based on the degree of N-deacetylation. The structures of chitin and chitosan and the naming convention are shown in the figure 06 **(THOMAS et al., 2020).**

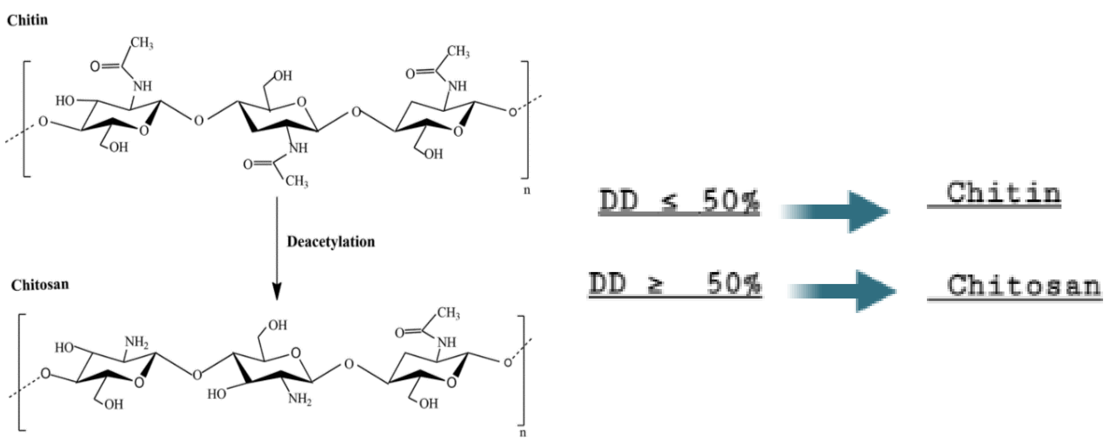


Figure 8 : Structure of chitin and chitosan (CASADIDIO et al., 2019)

3. Source of chitosan

Chitosan can be found in similar sources as chitin, such as aquatic organisms, insects, terrestrial crustaceans, mushrooms, and some fungi. However, from a production point of view, production of chitin typically involves marine crustaceans due to the abundance of low-cost by

products from the seafood processing industry. The shells of these crustaceans contain 15-40% chitin, with proteins (20-40%) and calcium carbonate (20-50%) as other major components. **(CRINI and LICHTFOUSE, 2019).**

4. Chitosan production

To extract chitosan, the chitin must first undergo deacetylation, which is typically achieved by chemical means. Chitin can be extracted from various sources, including crustaceans and shellfish waste, through demineralization, deproteination, and decolorization as already mentioned above. Once the chitin has been decolorized, deacetylation can be performed to obtain chitosan. Different methods can be used to achieve deacetylation **(AHMED and IKRAM, 2017).**

A- Deacetylation of Chitin by Chemical Method

Currently, there exist several techniques for the deacetylation of chitin, with the hot alkali treatment being the prominent method used in commercial and extensive applications. This method involves the utilization of sodium hydroxide (NaOH) for the deacetylation process **(THOMAS *et al*., 2020).**

The extent of deacetylation is influenced by various factors, such as the concentration of NaOH, reaction time, temperature, chitin or chitosan concentration, and molecular weight of the initial polymer.**(JENNINGS and BUMGARDNER, n.d.)**

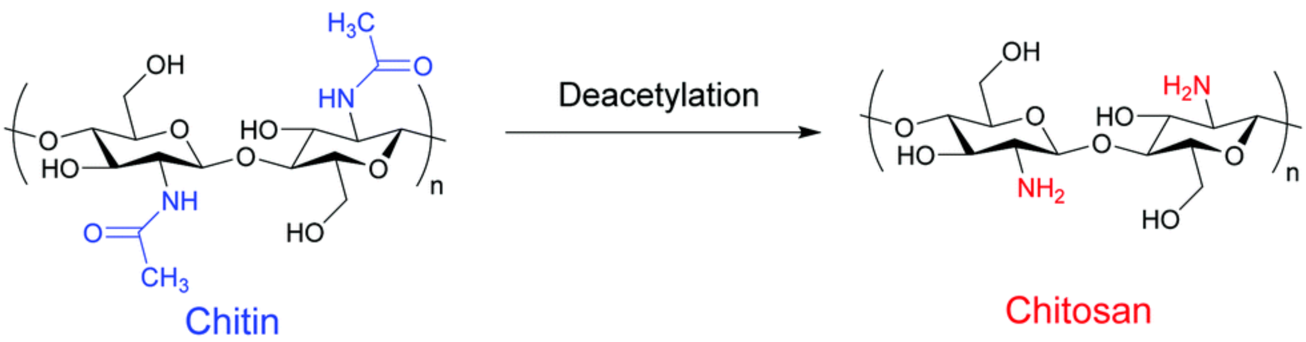


Figure 9 : Reaction scheme for alkaline deacetylation of Chitin (JENNINGS and BUMGARDNER, n.d.)

B- Deacetylation of chitin by enzymatic method

Aside from the alkaline deacetylation method, enzymatic deacetylation using chitin deacetylases derived from various biological sources, such as fungi and insects, offers a promising alternative for the production of chitosan derivatives **(MACKAY and TAIT, 2012)**.

This approach enables the control of enzymatic activity and minimizes depolymerization, resulting in a homogeneous product with a more defined pattern of deacetylation. Furthermore, enzymatic deacetylation is considered a more environmentally friendly option than thermochemical processes. However, this method can be cost-prohibitive due to the high cost of chitin deacetylases. Additionally, compared to the industrial chemical process, the enzymatic deacetylation method may yield a lower quality product at a higher cost. **(JENNINGS and BUMGARDNER, n.d.).**

C- Deacetylation of Chitin by Microwave Assisted Method

It has recently been discovered, that the three-step extraction process of chitin can also be accomplished through microwave-assisted mechanisms, providing a time-saving and environmentally friendly alternative**. (PELLIS *et al*., 2022).**

Knidri et al, demonstrated successful chitosan production through microwave-assisted deacetylation, achieving a degree of deacetylation of 82.73% within 24-minute timeframe. This result is noteworthy as it surpasses the degree of deacetylation attained through conventional methods, which require 5-10 hours to achieve a degree of deacetylation of 81.5%. **(AHMED and IKRAM, 2017)**

5. General Properties

Chitosan is a form of polysaccharide that consists of varying amounts of (1-4)-glycosidic bonds connecting glucosamine and N-acetyl-glucosamine. The presence of functional groups, including amino, acetyl amino, and hydroxyl groups in the chitosan sequence Fig (10), exhibits various physical-chemical, biological, and technological properties **(ARANAZ *et al*., 2021)**

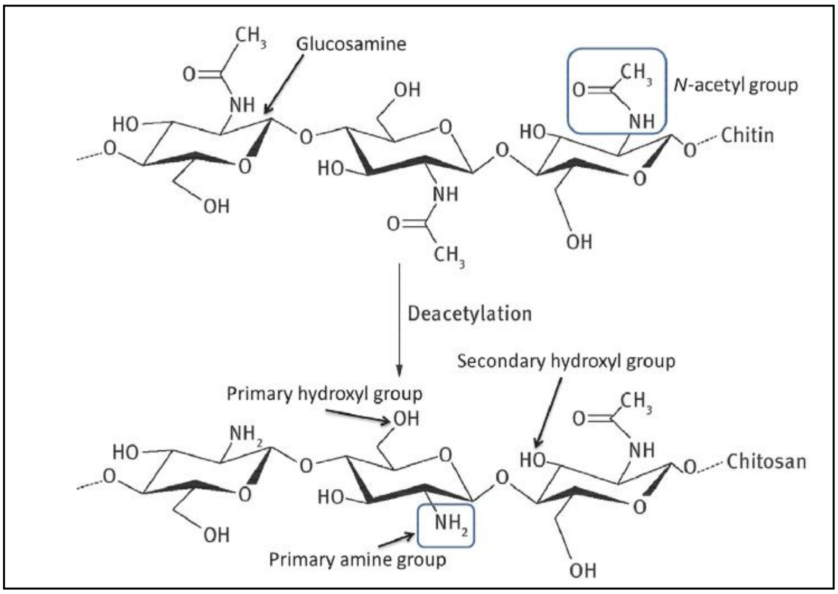


Figure 10 : Major units in chitin and chitosan (THOMAS et al., 2020)

Chitin and chitosan possess a variety of desirable characteristics that have made them among the most widely used biopolymers with numerous applications. These features include biocompatibility, biodegradability, adsorption capacity, antibacterial properties, gel-forming abilities, as well as the capacity to form films and chelate metal ions**. (SHUKLA *et al*., 2013) (ARANAZ *et al*., 2021).**

6. Physicochemical properties

A- Degree of Deacetylation (DD) of Chitosan

The degree of deacetylation is generally regarded to be a key factor of the structural parameters contributing to the physical and biological properties of chitosan. The degree of deacetylation identifies the biopolymer as chitin or chitosan. The DD of chitosan depends mainly on the source of the chitin and chitosan preparation process. In the case of chitosan, DD must be above 50%. **(HASAN *et al*., 2022),** The DD is an indicator of the efficiency of chemical deacetylation and the purity of chitosan, which increases with higher DD**. (HOSNEY *et al*., 2022).**

The degree of deacetylation (DD) of chitosan can be determined using various analytical methods, such as infrared spectroscopy, nuclear magnetic resonance spectroscopy, and elemental analysis. In addition, other methods such as titration, viscometry, and colorimetry also can be employed. **(NURHAYATI and TANG, 2022) (AHING and WID, n.d.) (SABNIS and BLOCK, 1997).**

B- Molecular weight

The molecular weight (MW) of a polymer is an important factor that determines the strength of its fibre and the viscosity of the solution. In the case of chitosan, the MW is closely related to the degree of deacetylation (DD), Studies have demonstrated that the MW of chitosan decreases as the degree of deacetylation increases **(HASAN *et al*., 2022).**

Based on their MW, chitosan can be classified into three categories **(THOMAS *et al*., 2020).**

Table 1 : Chitosan classification based on MW :

Low-molecular weight Chitosan	**MW < 50 kDa**
Medium-molecular weight Chitosan	MW 50-250 kDa
High-molecular weight Chitosan	MW > 250 kDa

Several methods are used to calculate the MW of chitosan, including light scattering, gel permeation chromatography (GPC), and capillary viscometry. Among these methods, capillary viscometry is considered the simplest and most widely used method for determining the MW of chitosan **(THOMAS *et al*., 2020).**

C- Viscosity

From a technological perspective, the viscosity of polymers is a critical parameter as highly viscous solutions can be challenging to handle and process. When chitosan is dissolved in an acidic solution, it yields a viscous solution. The viscosity of the solution is dependent on various parameters, including the molecular weight (MW), degree of deacetylation (DD), concentration, pH, and temperature of the chitosan solution. **(ARANAZ *et al*., 2021) (THOMAS *et al*., 2020).**

D- Solubility

The quality of chitosan is highly dependent on its solubility, as it is a crucial parameter that determines the purity of the chitosan produced. Research suggests that higher solubility is indicative of higher quality chitosan **(HOSNEY *et al*., 2022).**

The solubility of chitosan is influenced by several factors, including temperature, alkali concentration, deacetylation time, particle size of chitin, and the ratio of chitin to alkali solution. **(NURHAYATI and TANG, 2022).**

E- pH of Chitosan Solution

The pH of dilute acidic chitosan solutions is typically below 6.0. Although chitosan is a strong base due to the presence of free –NH2 groups throughout the chain, with a pKa value of 6.3, the pH of the solution can affect the properties and charge of chitosan as illustrated in the table. **(AHMED and IKRAM, 2017).**

Table 2 : The effect of PH on Chitosan solution

At low pH	At high pH values above 6.0	Reference
> The free –NH2 groups become protonated and acquire a positive charge, rendering chitosan a cationic polyelectrolyte and water-soluble. Moreover, it can form quaternary salts with nitrogen at low pH values	> The amino groups become deprotonated, resulting in the insolubility of chitosan	> (Ahmed and Ikram, 2017)

F- Water-binding capacity and fat-binding capacity

Chitosan's water and fat binding capacities are critical factors that determine its potential application as a food ingredient. The water binding capacity of chitosan is attributed to the presence of hydrophilic -OH and -NH2 groups in its structure, while the fat binding capacity is due to its amphiphilic nature **(THOMAS *et al*., 2020).**WBC and FBC are calculated as follows:

$$WBC\ \% = \frac{Water\ bound\ \ (g)}{initial\ sample\ weight\ (g)}$$

$$FBC\ \% = \frac{Fat\ bound\ (g)}{initial\ sample\ weight\ (g)}$$

Table 3 : WBC and WFC % References (THOMAS et al., 2020)

	Chitin %	Chitosan %
WBC %	381 to 673 %	458 to 805%
FBC %	316 to 320 %	314 to 535 %

7. Biological properties

Chitosan, a biopolymer obtained from chitin, manifests a plethora of biological properties that position it as a material with great potential for various applications **(AHMED and IKRAM, 2017)**. Among its significant attributes are the following:

Chitosan is a biocompatible material, due to its inherent biodegradability to normal body constituents, non-toxicity, and ability to support cell adhesion and proliferation **(PELLIS *et al*., 2022).** Its cationic nature allows for interactions with negatively charged molecules such as cell membranes and extracellular matrix components, promoting cell attachment and growth **(MOGOSANU and GRUMEZESCU, 2014)**. Moreover, chitosan's chemical structure can be modified to enhance its biocompatibility by altering its molecular weight, degree of deacetylation, and charge density table**. (HECTOR *et al*., 2016) (AHMED and IKRAM, 2017).**

Furthermore, its positively charged amino groups enable it to exhibit antibacterial, antiviral, and hemostatic activities **(SHUKLA *et al*., 2013)**. It also has fungistatic and antioxidant properties, as well as antitumor and immunoadjuvant activities **(ARANAZ *et al*., 2021) (MACKAY and TAIT, 2012).**

Table 4 : Influence of DD and MW of polysaccharidic formulation on biological activities (CASADIDIO et al., 2019)

Effect on Biological Activity	PhysicochemicalProperty
Antimicrobialactivity	↑ DD and ↓ MW
MW Antioxidantactivity	↑ DD and ↓ MW
Mucoadhesiveproperties	↑ DD and ↑ MW
Penetrationenhancementproperties	↑ DD increased activity, MW is not discriminating

Therewith Chitosan's manifold properties enable its facile conversion into diverse forms, rendering it suitable for a wide range of applications as shown in the Figure below:

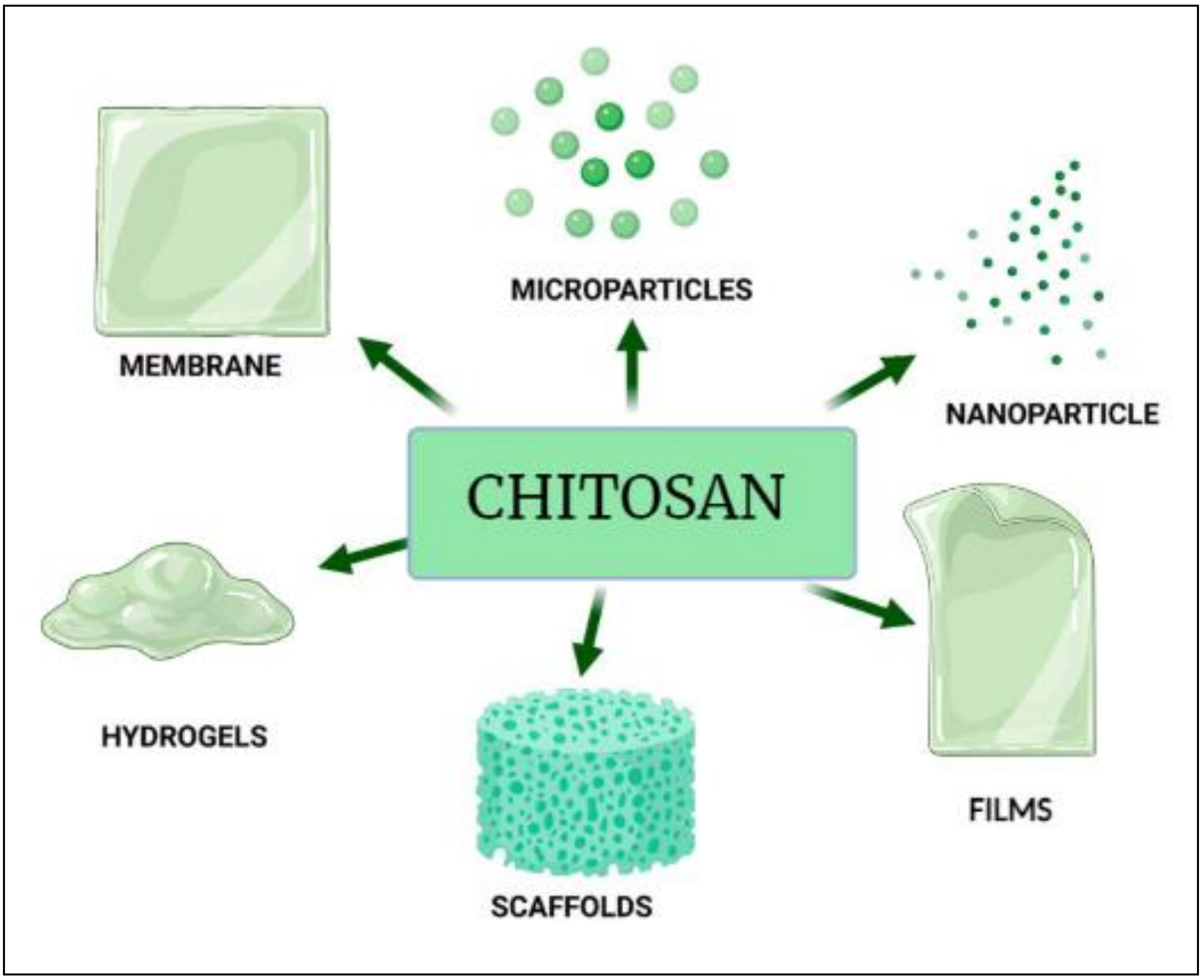


Figure 11:Schematic representation of the possibilities of processing chitosan into different forms

8. Chitosan derivatives

Chemical modification has the potential to augment the biological and physicochemical characteristics of chitosan **(AHMED and IKRAM, 2017).** Chitosan, despite its advantageous properties, is beset by the inadequacy of solubility in water and conventional organic solvents. However, this limitation can be resolved via chemical alteration **(THOMAS *et al*., 2020).**

One prominent example of a chitosan derivative is chitosan hydrochloride, which has gained significant recognition and inclusion in European Pharmacopeia 6.0 and the 29th edition of the

United States Pharmacopeia (USP) 34-NF. It exhibits remarkable solubility in water, rendering it highly versatile and suitable for a wide array of applications(**FOURNIER *et al*., 2020**) **(ABD EL-HACK et al., 2020), (ARANAZ et al., 2021).**

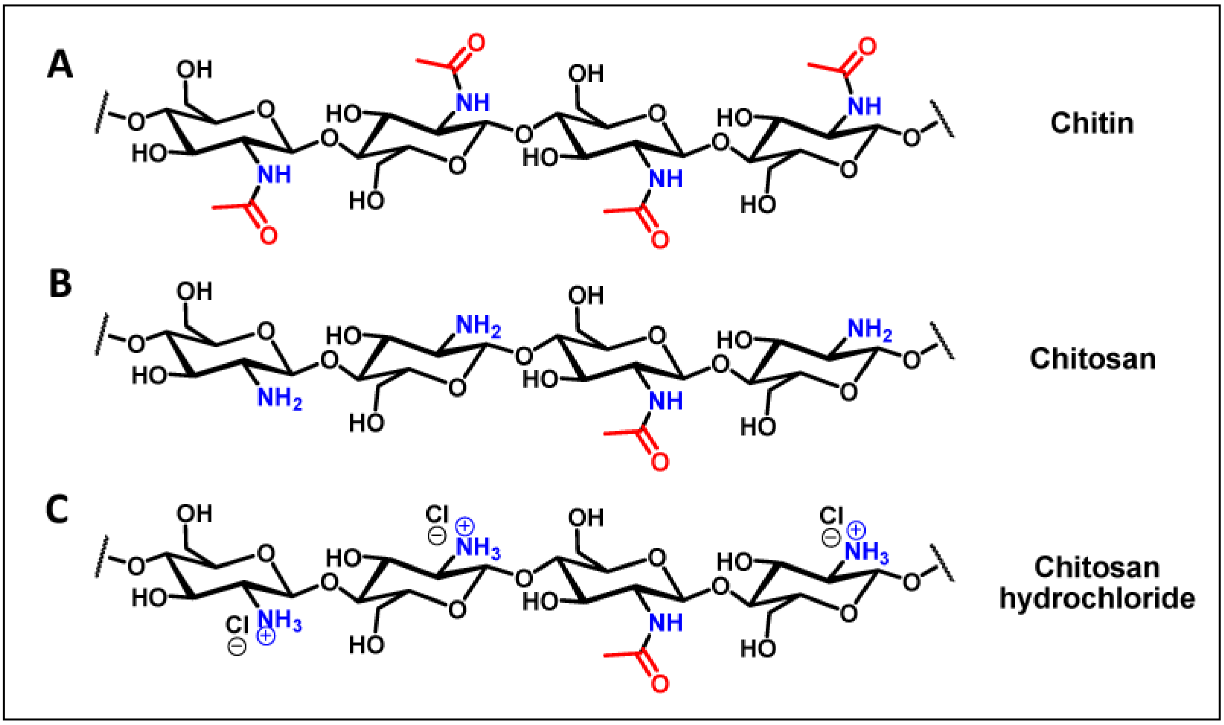


Figure 12:Chemical structure of Chitin (A) chitosan (B) and Chitosan Hydrochloride (C) (FOURNIER et al., 2020).

Table 5:Generalties about Chitosan hydrochloride

Generalities	Properties	Application
• known under the names: Chitosan hydrochloride, Chitosan chloride, Chitosanihydrochloridum • listed in Eur. Ph., for medical applications • soluble in water	• Stronger swelling behaviour than chitosan • Water soluble • Hemostaticeffects • Mucoadhesion • Increases the permeability of tight junctions in the intestine.	• Hydrogels • Wound dressings • Drug transport and release • Spray Solutions

III. Characterization of chitin and chitosan

Fourier transform infrared spectroscopy (FTIR) is a powerful analytical technique used to investigate the functional groups and chemical bonding in a wide range of materials.

In the case of chitin and chitosan, FTIR analysis provides valuable information about the chemical composition, purity, and degree of deacetylation of the samples. The FTIR spectra of chitin and chitosan are characterized by distinct absorption peaks corresponding to specific functional groups, such as carbonyl, amide, and hydroxyl groups. These peaks can be used to identify and quantify the different components of the samples and to monitor changes in their chemical structure and composition under different conditions**. (THOMAS *et al*., 2020)**

The following tables provide a visual representation of the characteristic IR-bands exhibited by chitin and chitosan samples by (**THOMAS *et al*.,2020)**.

Table 6 : Characteristics IR-bands of chitin samples

Frequency (cm−1)	Assignment
850–900	CH & COC stretching
1000–1050	COC stretching
1100–1150	Asymmetric ring stretching
1300–1320	Amide III band
1400–1420	CH2 bending
1550–1560	Amide II band
1620–1630	Amide I band
1640–1660	Amide I band
2850–2900	CH stretching
2930–2950	CH2/CH3 stretching
3250–3270	NH stretching
3400–3450	OH stretching

Table 7 : Characteristics IR-bands of chitosan samples

Frequency (cm−1)	Assignment
1030–1070	COC stretching
1150–1160	Asymmetric ring stretching
1310–1330	Amide III band
1420–1430	CH2 bending
1540–1560	Amide II band
1580–1600	Amine groups bending
1640–1660	Amide I band
2850–2900	CH stretching
3250–3300	NH stretching
3400–3500	OH stretching

IV. Chitosan application

The utilization of chitosan as a biopolymer has garnered significant attention, particularly in domains where safety is essential, such as biomedicine, pharmacy, cosmetics, agriculture, and food packaging industries (Figure 13).

In all of these domains, the potential application of chitosan is intrinsically linked to the human body. Chitosan's biocompatibility and non-toxicity constitute crucial attributes that render it an attractive material for deployment in the aforementioned applications. **(SINGH DHILLON *et al*., 2013).**

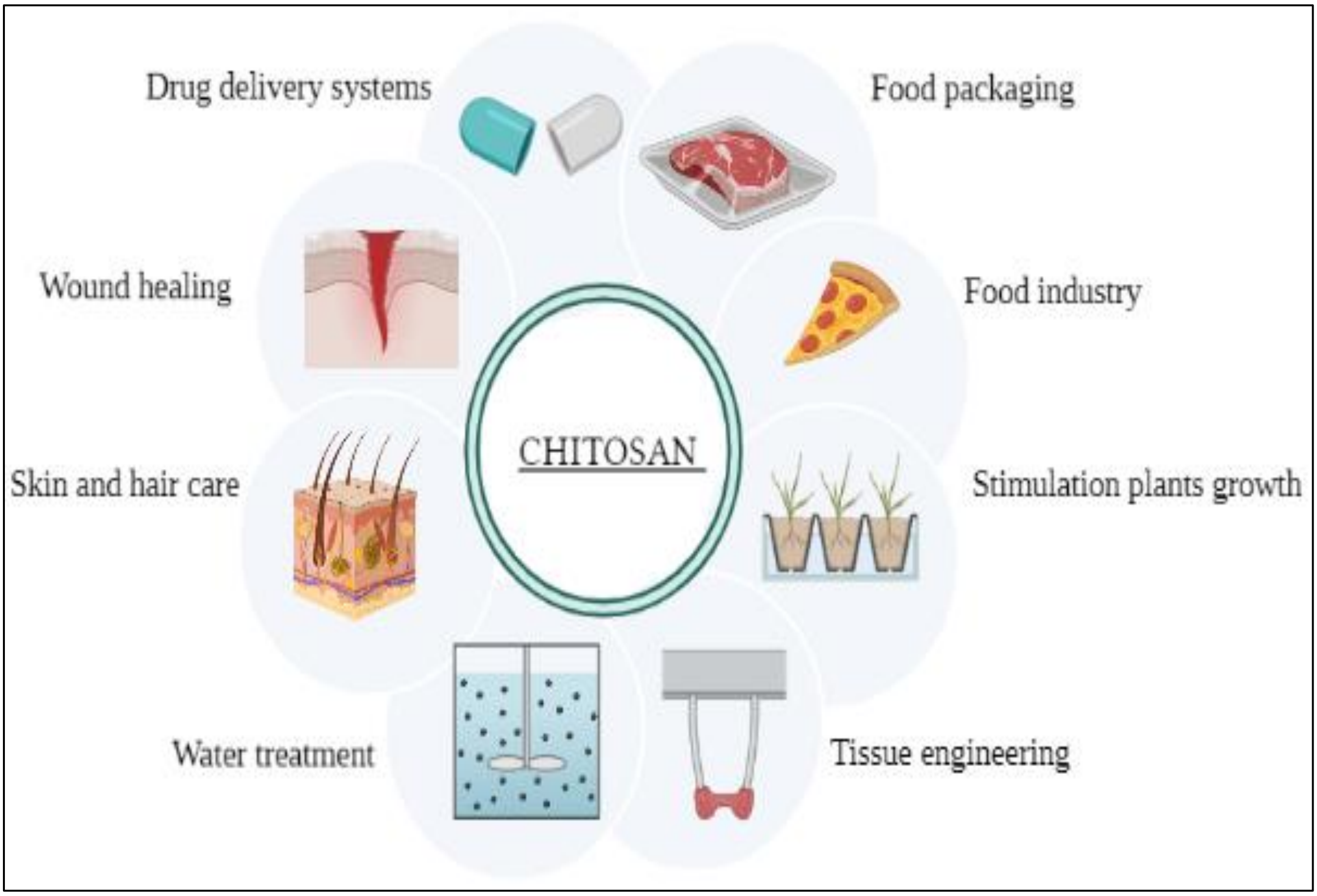


Figure 13 : Various application of Chitosan

1. Chitosan in Biomedicine

Chitosan, a polycationic polymer, has been the subject of extensive investigation in the fields of pharmaceutics and biomaterials. Its usage offers several advantages that make it particularly attractive for these applications **(MACKAY and TAIT, 2012)**. Firstly, chitosan is a natural product derived from crustacean shells containing chitin, which is widely abundant in nature. Also, it is enzymatically degradable, providing an added benefit in terms of its biocompatibility. Chitosan is easily functionalized and complexable, making it an attractive candidate for various applications, it exhibits a relatively low toxicity compared to most polycations, making it comparable to more expensive synthetic polycations. Nevertheless, it is important to note that chitosan toxicity has been reported in animals, with a LD50 of 16 g/kg body weight in laboratory mice, which is similar to that of salt or sugar (**ZARGAR *et al*., 2015).**

Table 8: Applications of chitosan in biomedicine and pharmaceuticals (SINGH DHILLON et al., 2013).

Properties	Efficacy
High antibacterial and antifungal activity	Inhibits pathogenic bacteria and fungal growth. Being biocompatible with human tissues CTS is used as natural cicatrizant.
Immunization	Enhances the body's immunity to diseases in humans. Actssimilarly in plants.
Anticholesterolemic effect	Helps to lower the level of cholesterol.
Haemostatic action	CTS and its derivatives, such as sulphated CTSs possess high anticoagulant activity.
Salt adsorption	Lowers high blood pressure.

2. Antimicrobial - antifungal activity of Chitosan

Chitosan demonstrates strong antimicrobial activity against various microorganisms, including Gram-positive bacteria, Gram-negative bacteria, and fungi **(KE *et al*., 2021).** Its effectiveness as an antimicrobial agent depends on factors such as the type of pathogen, pH level of the environment, and specific structural properties like degree of deacetylation, molecular weight, source, and concentration. Moreover, marine-sourced chitosan has been found to have higher activity compared to chitosan derived from the fungus. **(ABD EL-HACK *et al.*, 2020).**

The mechanisms of action of chitosan against bacteria and fungi

Gram-positive and Gram-negative bacteria possess notable disparities in their cell wall structures. Gram-positive bacteria are characterized by thick peptidoglycan layers, while Gram-negative bacteria are rich in lipopolysaccharides (LPS). These distinctions in cell surface structure contribute to divergent susceptibilities to chitosan. **(KE *et al.*, 2021).**

Specifically, Gram-negative bacteria typically exhibit a higher negative charge owing to the presence of phosphorylated groups associated with LPS (Figure 14-B). This increased negativity facilitates the binding of cationic chitosan to the phospholipids on the cell, in the other hand, Gram-positive bacteria, possess a thick cell wall that can impede direct binding of chitosan to the cell membrane. Nevertheless, certain chitosan oligomers with a molecular weight below 5 kDa are capable of penetrating the cell wall and influencing processes such as DNA/RNA or protein synthesis. (Figure 14-A) **(SANTOS *et al.*, 2020).**

Furthermore, Chitosan has been shown to have fungicidal effects on several fungal pathogens in plants and humans. Its antifungal properties are mainly related to the interaction of chitosan with the cell wall or cell membrane (Figure 14-C) **(ABD EL-HACK *et al.*, 2020).**

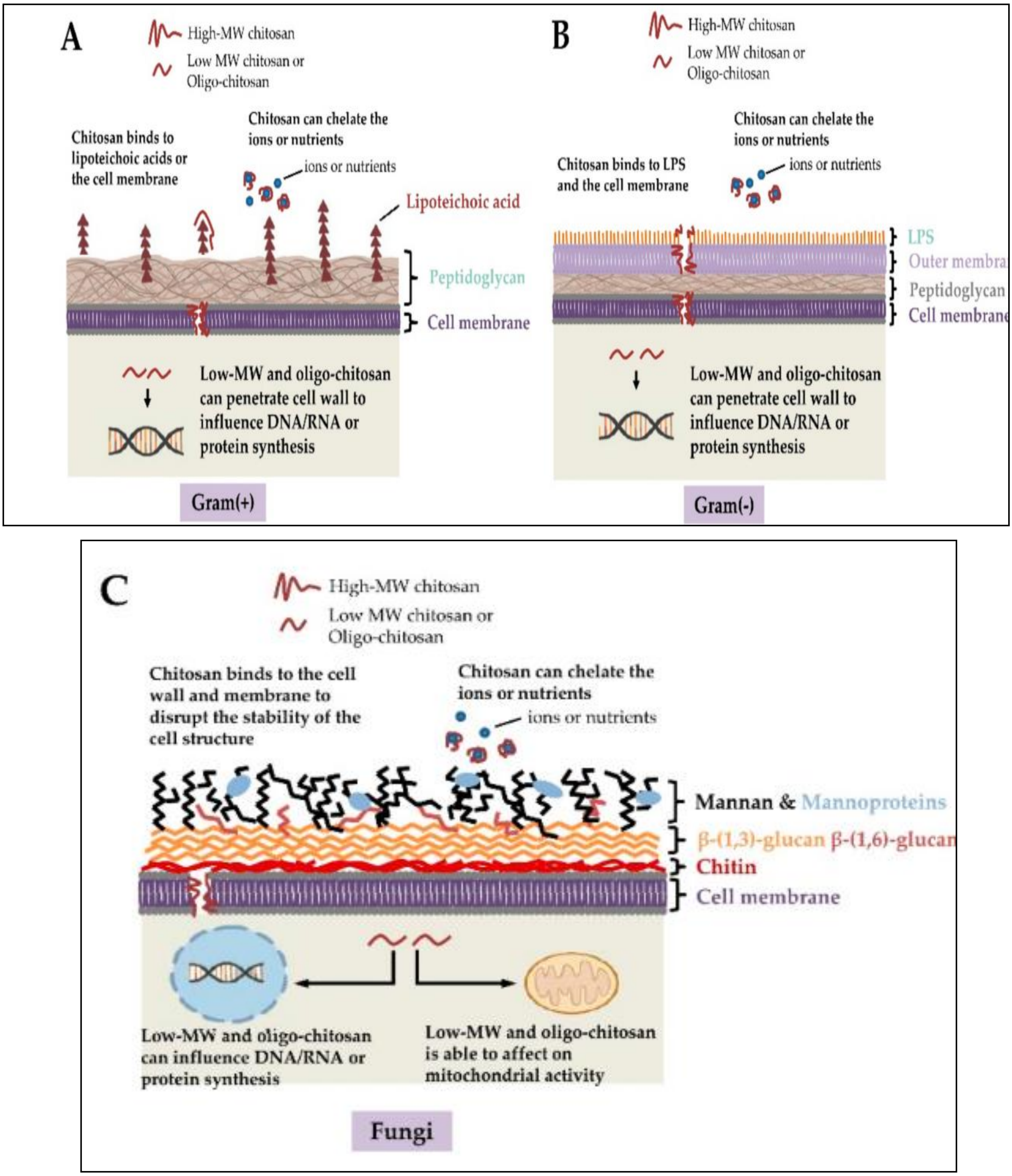


Figure 14 : Potential antimicrobial actions of chitosan against (A) gram-positive bacteria, (B) gram-negative bacteria, and (C) fungi. (KE et al., 2021)

3. Chitosan in Drug Delivery

The utilization of polymers in drug delivery has been a significant advancement in the field of biomedicine and industry. Amongst the various biopolymers investigated for these applications, chitosan stands out owing to its superior biological and physiochemical properties. The distinctive feature of this biopolymer is its cationic character attributed to its amino groups. This cationic nature is the basis for a plethora of properties exhibited by chitosan, including controlled drug release, mucoadhesion, in situ gelation, transfection, permeation enhancement, and efflux pump inhibitory properties. **(AHMED and IKRAM, 2017).**

Initially, chitosan hydrochloride was approved in 2002 by the Pharmacopeia. Chitosan was first introduced as an excipient into the European Pharmacopeia 6.0 and the 29th edition of the United States Pharmacopeia (USP) 34-NF almost ten years later. The increase in the number of publications regarding the use of this polymer in drug delivery is shown in Figure , and reveals a strong increase since 2002 that is still maintained today. **(ARANAZ *et al*., 2021).**

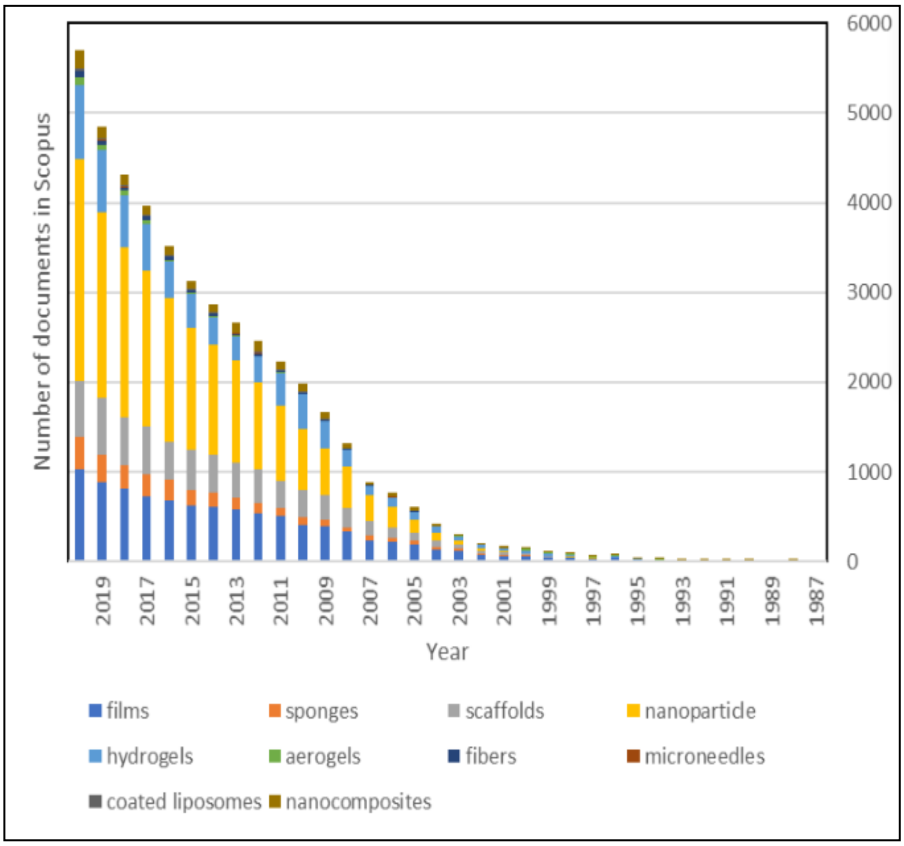


Figure 15 : Publications about chitosan drug delivery in Scopus (1987–2020).

4. Wounds and burns healing

The skin, which constitutes about 15% of our body weight, plays a vital role in regulating body temperature, preventing water loss, and shielding internal organs from the environment. It consists of three main layers: the epidermis, dermis, and hypodermis (Figure 16), The goals of wound healing are preventing infection, maintaining a moist environment, wound protection, and minimum scar formation. **(HASAN *et al*., 2022).**

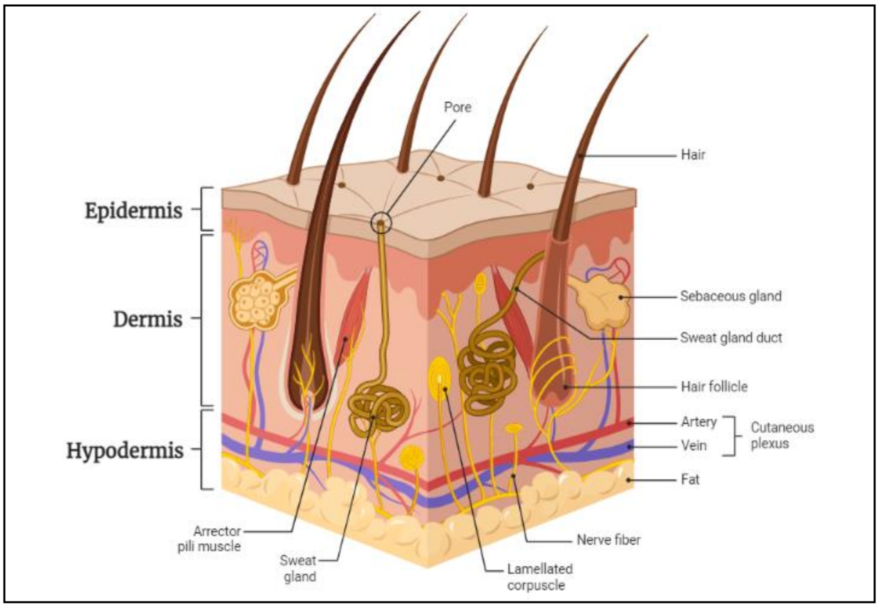


Figure16 : Skin anatomy

Chitosan, a derivative of chitin found in the exoskeleton of crustaceans, has emerged as a promising candidate due to its biocompatibility, antimicrobial properties, and ability to accelerate tissue regeneration. Chitosan-based products have been extensively studied for their applications in wound healing, showing promising results in various preclinical and clinical studies. **(HASAN et al., 2022) (KORDESTANI, 2019) (ŞENEL and MC-CLURE, 2004).**

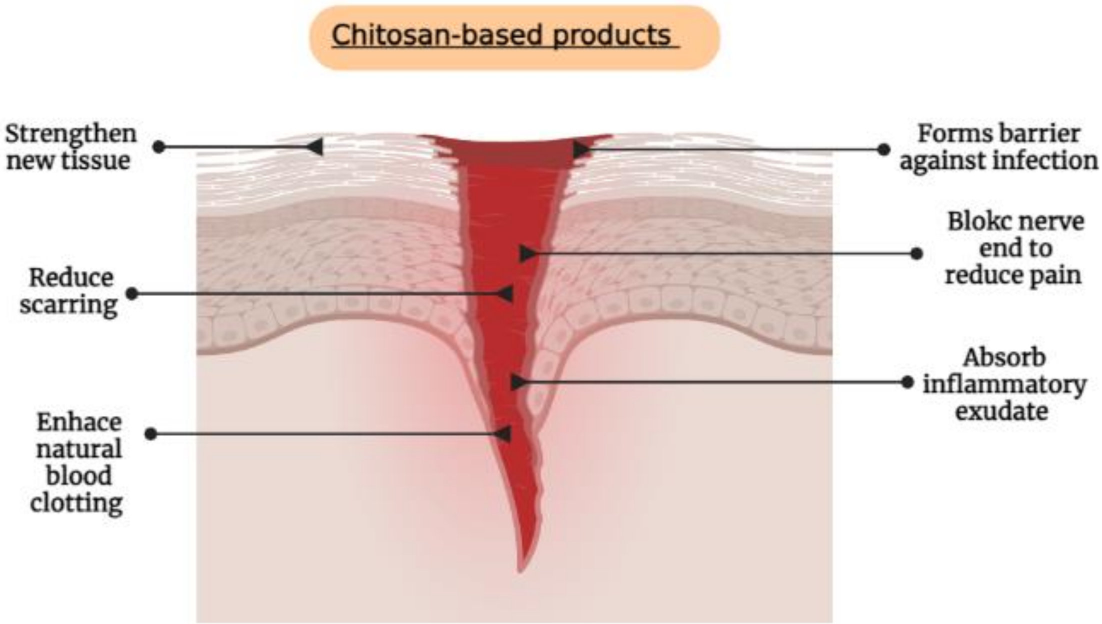


Figure 17 : Mechanisms of wound healing promotion by chitosan-based products.

Chitosan-based hydrogels and composite hydrogels have been successfully utilized for wound care, effectively cleansing wounds and burns, reducing pain, and decreasing temperature (**KORDESTANI, 2019).**

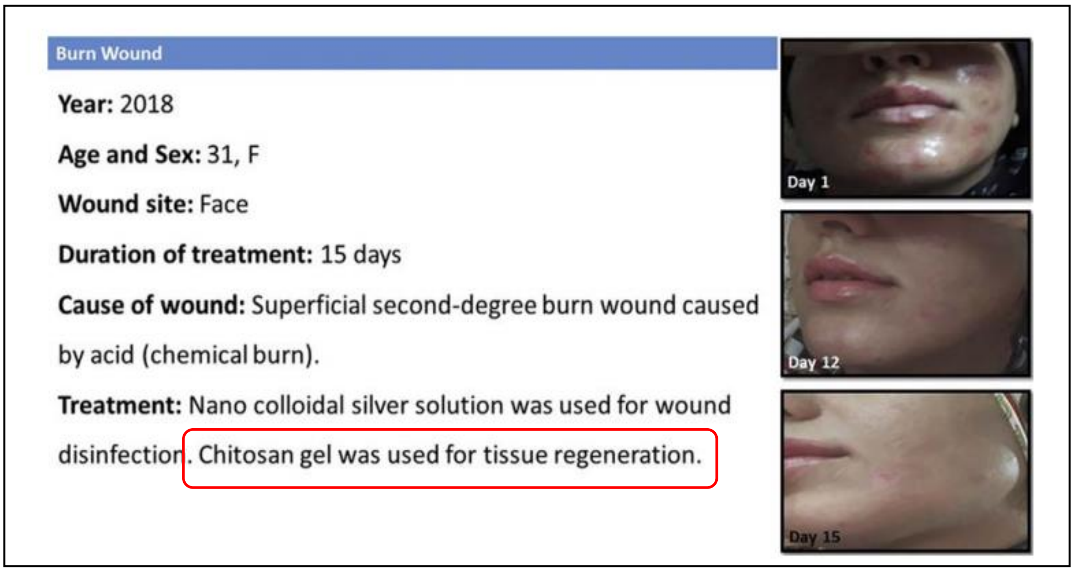


Figure 18 : Superficial second-degree burn treated with chitosan gel (KORDESTANI, 2019)

These hydrogels can also be designed to incorporate various therapeutic substances, including proteolytic enzymes and antibiotics, to accelerate the healing process. Additionally, chitosan-based bandages have demonstrated exceptional bactericidal and haemostatic properties, making them a preferred choice for the US army and UK military in wound management. **(AHMED and IKRAM, 2017)**, A number of these formulations has come out to the market, like **Beschitin** in Japan (based on chitin) or **Hem Con** in USA (based on chitosan), **(ZARGAR et al., 2015).**

Figure 19 : Market products based on chitin and chitosan

Studies have shown that the use of chitosan-based polymers and products can expedite wound healing, reduce the need for frequent treatments, and provide comfortable and pain-free protection to the wound surface. Furthermore, chitosan has been found to activate the body's natural defence mechanisms, effectively preventing infections and offering an alternative to antibiotics. **(ŞENEL and MC-CLURE, 2004) (KORDESTANI, 2019).**

5. Additional cosmetics properties

Within the category of polysaccharides, chitin, chitosan, and their derivatives possess inherent attributes associated with antiaging, inhibition of matrix metalloproteinases (MMPs), antioxidant capabilities, and antifungal properties. These distinctive qualities make chitin and chitosan viable candidates for employment in diverse domains encompassing skincare, oral hygiene, nail care, and hair treatment. Consequently, the development of formulations incorporating chitin and chitosan has emerged as a potential avenue for addressing various ailments affecting the teeth, hair, nails, and skin. **(CASADIDIO *et al.*, 2019).**

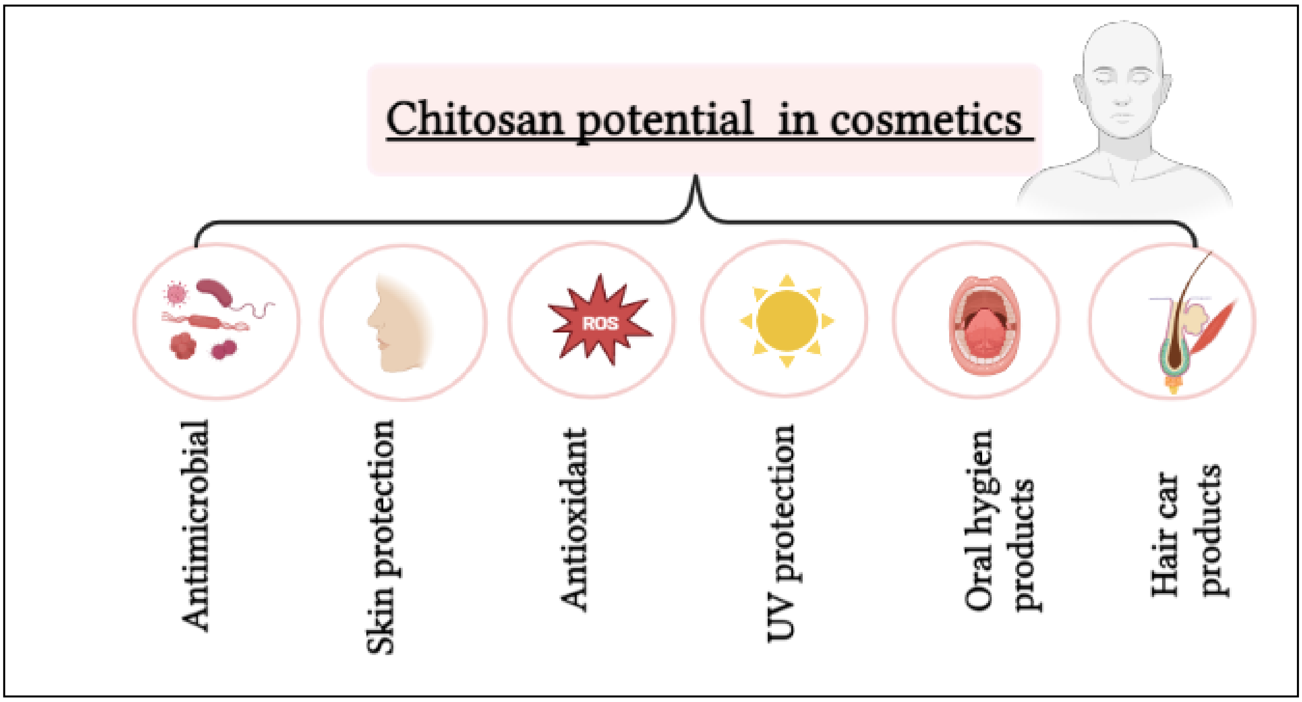


Figure 20:Chitosan application in Cosmetics

6. Paper and textile industry

Chitin and its derivative, chitosan, exhibit similarities to cellulose, making them suitable for reinforcing recycled paper and packaging materials. This characteristic reduces the need for excessive chemical additives while delivering enhanced outcomes such as improved surface smoothness and moisture resistance. Specifically, hydroxymethyl chitin finds application in the manufacturing of toilet paper and wrapping paper Additionally, chitin and its derivatives have been utilized in various areas, including the development of antistatic and soil-repellent materials, printing and finishing agents, dye removal agents, and the production of textile sutures, threads, and fibres. These versatile applications highlight the wide-ranging potential of chitin and its derivatives in various industries. **(THOMAS et al., 2020).**

7. Agriculture

Chitosan, has gained significant attention for its potential applications in agriculture and plant protection. With its unique properties such as biodegradability, biocompatibility, and non-toxicity, chitosan offers a promising alternative to conventional synthetic chemicals used in crop management. Studies have demonstrated that chitosan can effectively enhance plant growth, improve nutrient uptake, and stimulate the plant's natural defense mechanisms against various pests and diseases. Furthermore, chitosan has shown efficacy in controlling fungal, bacterial, and viral pathogens, making it a valuable tool for sustainable crop protection. Its ability to induce systemic acquired resistance in plants and activate plant defense-related genes further strengthens its potential as a bio-based agricultural solution. As research in this field continues to expand, exploring the multifaceted benefits of chitosan in agriculture holds great promise for promoting environmentally friendly and sustainable practices in plant protection. **(EL HADRAMI *et al*., 2010**

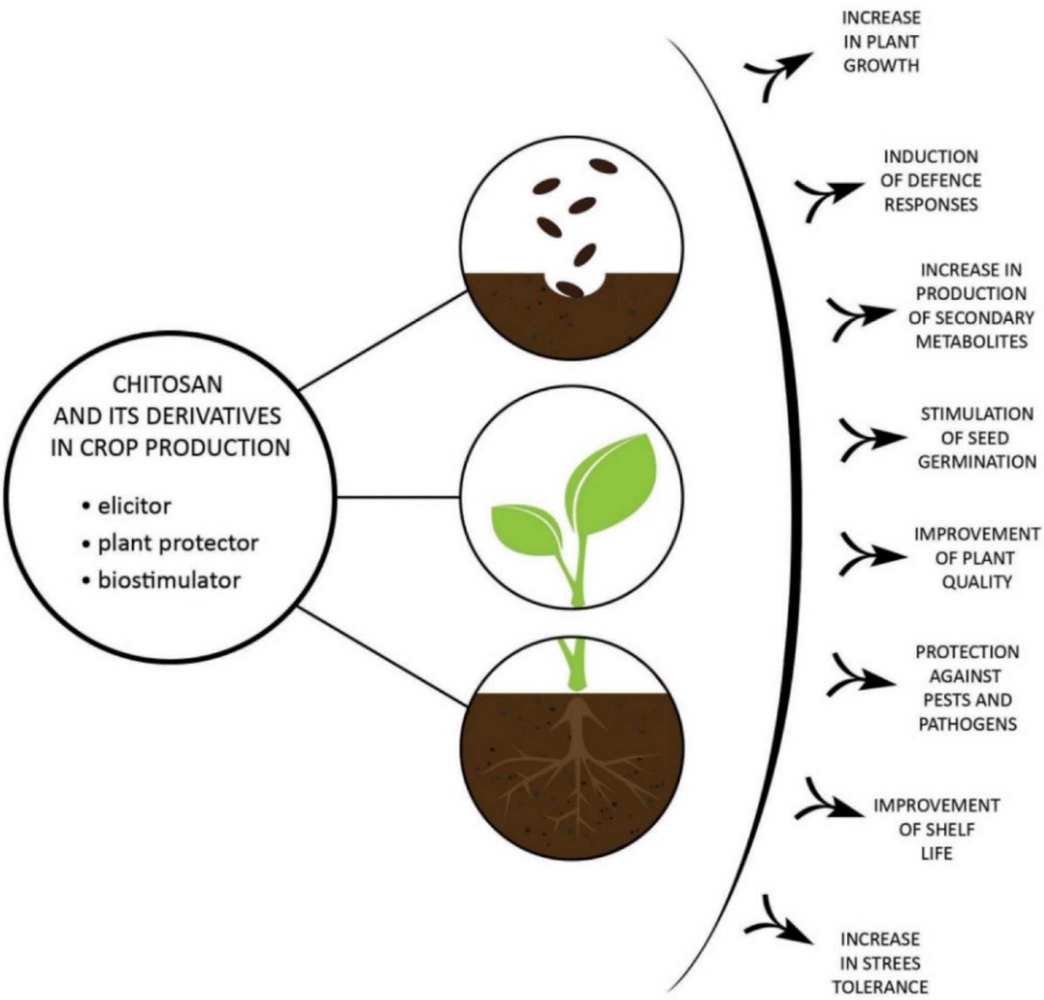


Figure 21:Application of chitosan and its derivatives in plant production

(STASIŃSKA-JAKUBAS and HAWRYLAK-NOWAK, 2022)

II. Experimental part

I. Material

1. Biological Material

The biological material used in the present study is:

- Shrimp shell waste (10 kg)
- Pathogenic germs:
  - *Escherichia coli*
  - *Staphylococcus aureus*
  - *Pseudomonas aeruginosa*
- Fungus
  - *Candida albicans*
- Spider plants (*Chlorophytum comosum*)
- Commercial chitosan (RUIHERBS)
- Egg white
- Domesticated albino Rabbit (*Oryctolagus cuniculus*).

2. Non-Biological Material

The apparatus and reagents that were used in this study are listed in this table below:

Table 9: Apparatus and reagent used in this study

Apparatus	Reagent
• BALANCE	• ACETIC ACID
• BALLONS	• ACETONE
• BAKERS	• ALCHOOL BENZYLIC
• BOROSILICATE FLASH	• ALCHOOL CETO-STEARYLIC
• BUNSEN BURNER	• CETYLIQUE ALCHOOL
• CAPILARY RULER	• STEARYL ALCHOOL
• CENTRIFUGE	• AMMONIA
• CENTRIFUGE TUBES 50ML	• AMMONIUM
• COFFEE GRINDER MACHINE	• AMMONIUM THIOCYANATE
• COTTON DISKS	• BLEACH
• COTTON SWABS	• CETYL PALMITATE
• DIGITAL ROTARY VISCOSIMETR	• COOPER SULPHATE
• DISSICATOR	• FERRIC SULPHATE
• ERLENMEYER FLASK	• GLYCERIN
• FILTRATION MEMBRANE	• HCL
• FORCEPS	• ISOPROPYL MYRISTATE
• FREEZER	• LIQUID PARAFFINE
• FT-IR SPECTRUM	• METHYL ORANGE INDICATOR
• GLASS FILTRES	• METHYL SALICYATE
• GLASS FUNNEL	• MONOSTEARATE GLYCEROLE
• GRADUATED TEST TUBES	• MONOSTEARATE GLYCEROLE
• HEATED MAGNETIC STIRRER	• MUELLER HINTON AGAR
• INCUBATOR	
• INOCULATING LOOP	
• MAGNETIC BARS	

• MUFFLE FURNACE • OVEN • PETRI PLATES • PH METER • PIPETTE • PORCLAIN CRUCIBLE • RACK • REFLUX REFRIGERANT • ROUND-BOTTOM FLASKS • SCISSORS • SEIVE OF (200 UM) • SPATULAS • STRAINER • THERMOMETRE • TUBE TESTS • VACCUM OVEN • VACCUM PUMP • VOLUMETRIC FLASK • VORTEX • WASH BOTTELS	• MYRISTATE • NAOH PALLETS • NITRIC ACID • NITROGEN • POLYSORBATE 60 • PROPYLENE GLYCOL • PURIFIED WATER • SILLICA GEL BLEU • SILVER NITRAT • SODIUM AND POTASSIUM TARTRATE • SORBITOL MONOSTEARATE • STEARATE MACROGOLA • TAP WATER

II. Raw material collection and processing

1. Source of the raw material

The raw material “shrimp shells waste “was collected at the end of December 2022, from different restaurants located in Bab Ezzouar, the raw material was composed of different types of shrimp waste, the approximate value of the total masse is about 10 kg.

2. Shell pre-treatment

Before initiating the use of the sample, some steps are considered essential to obtain the desired result which are described in three points:

I. Cleaning process

This step aims to eliminate any trace of flesh, sand residues, microorganisms and other substances that may be attached on the surface, using running tap water several times

Figure 22:Shrimp shell waste cleaning process

II. Drying process

The shrimp shell was dried in the open air, away from direct sun light, placed on cooking paper for a period of 2 to 5 days, in order to get rid of the majority of water in the shell until obtaining a crispy crust, The sample was stored in a closed container prior to use.

Figure 23 : Shrimp shell drying process

III. Grinding and sieving process:

The dried material was grinded by electric coffee grinder machine until a thin powder was obtained, after that, the sample has been sieved by 200 µm sieve to obtain a thin consistent powder.

The amount of powder obtained at the end of the grinding process is about 400 g.

Figure 24 : Grinding and sieving of shrimp shells

The purpose of performing raw material treatment on shrimp shell waste through cleaning, grinding, and sieving is to extract high-quality chitin and chitosan.

By cleaning the waste, the impurities can be removed, and the subsequent grinding and sieving processes can produce a purified and homogeneous material suitable for chitin and chitosan extraction. This raw material treatment process ensures the production of high-quality chitin and chitosan with optimal purity, which can then be utilized in various applications.

III. Chitin and chitosan production by chemical methods

The conventional approach to chitin extraction from crustacean exoskeletons involves two main steps. Initially, the protein is separated via alkali treatment, followed by separation of calcium carbonate (and calcium phosphate) through acidic treatment at high temperatures. Subsequently, the resultant product undergoes a bleaching process using chemical reagents to achieve a colorless final product.

The primary technique utilized to obtain chitosan involves alkaline deacetylation of chitin using a potent alkaline solution for varying durations.

Methods (Extraction of Chitosan)

In this study, the production of chitin and chitosan was carried out based on three protocols described in the literature, with modifications made after numerous trials to achieve a high extraction yield and a high-quality chitosan product.

Method 01

This method is conducted according to a private document by European Science and Technology in Action Building Links with Industry, Schools and Home.

Protein removal:

15 g of the dried shells are transferred into an Erlenmeyer flask.

250 ml of sodium hydroxide solution 2% are added and the mixture is heated under stirring at 60 - 70 °C for half an hour, The shells are filtered off with a strainer and the process is repeated, the filtrate should be almost clear and colorless. Then the shells are washed to neutral with demineralized water.

For time-saving the shells are soaked in sodium hydroxide solution overnight, after the first sodium hydroxide treatment, then filtered off and washed

Figure 25 : Method 01- protein removal process

Calcium carbonate removal:

250 ml of hydrochloric acid 7% are slowly added to the shells and the mixture is stirred at room temperature until no gas escapes. As a check, 10 ml of hydrochloric acid are added. If no generation of gas occurs, the mixture is filtered off and washed neutral with water. The product is dried overnight in the oven at 60 °C.

Figure 26:Method 01 - calcium carbonate removal

Chitosan from chitin by alkaline hydrolysis:

In a round-bottom flask 150 ml sodium hydroxide solution 50% are added to 2 g of chitin. The apparatus is flushed with nitrogen and then it is closed air-tightly with a nitrogen-filled balloon. Now the mixture is heated under stirring to at 125°C for one hour. The mixture is allowed to cool and then 100 ml of water are added. The next day the mixture is filtered off and the residue is washed with water to neutral reaction and dried in the oven at 60°C.

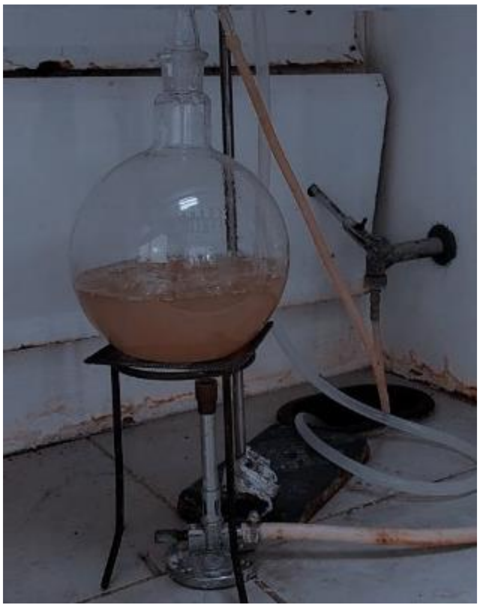


Figure 27 : Method 01- alkaline hydrolysis of chitin

Method 02

This method was conducted according to **(KANDILE *et al*, 2018)** with modifications.

Protein removal:

The deproteinization process was carried out by weight 15g of shrimp shells powder by using 5% NaOH with a weight to volume ratio of 1g:8ml (w/v).

The solution with shrimp shells was refluxed at 70˚C for 3 h, The product was collected and washed until clear solution, it was then dried in a vacuum

The product was decolorized with pure acetone for 24 h, then collected and washed to neutrality, then dried.

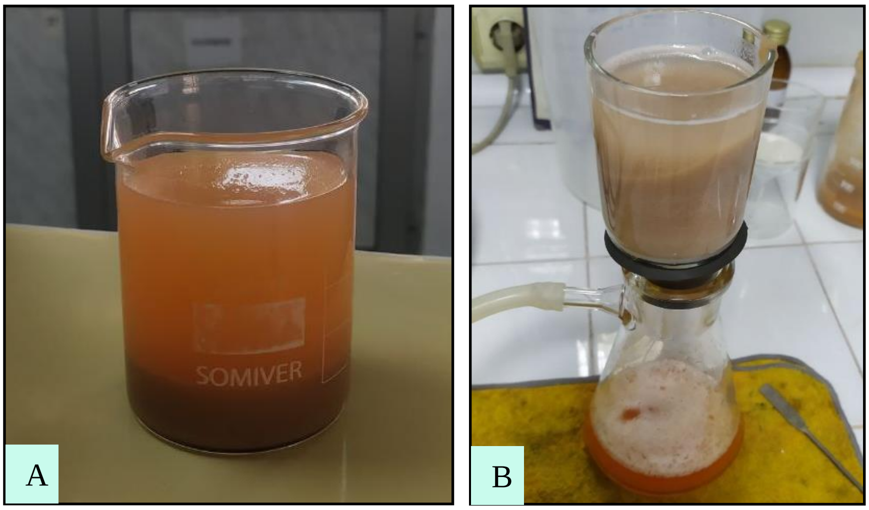


Figure 28 : Method 02 – bleaching (A) and washing process (B)

Calcium carbonate removal:

The decolorized product was demineralized by using 1% HCl, with a weight to volume ratio of 1g:10ml for 24 h at room temperature. The product was collected and washed to give light brown powder.

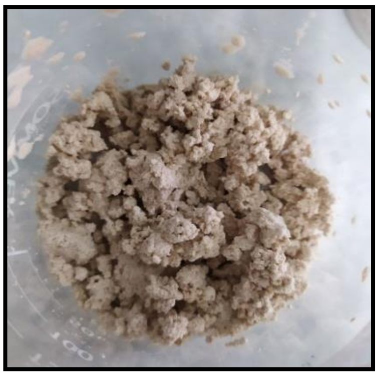


Figure 29 : Method 02 -resulting chitin powder

Chitosan from chitin by alkaline hydrolysis:
The N-deacetylation of the demineralized product was carried out by using 55% NaOH, with weight to volume ratio of 1g:5ml at 100˚C for 12 h. The product was washed with distilled water and dried overnight.

Figure 30 : Method 02 - alkaline hydrolysis (A) and washing process

Method 03

This method was conducted by **(AHING AND WID, 2016)** with modifications.

Protein removal:

A total of 15 g samples of raw shrimp shell waste were added with 2.0 M NaOH in the ratio 1:16 (w/v) then left for 48 hours at room temperature, with pH ranged from 11-13.

After that, the solution was filtered and the samples were washed with distilled water until neutral pH was achieved (pH6.5-8.0). Water from the samples was removed before performing the demineralization process.

Figure 31 : Method 03- deproteinization process

Calcium carbonate removal:
Samples from deproteination process were added with 1.0 M HCl in the ratio 1:16 (w/v) and allowed to stand for 24 hours, with pH value ranged pH 1.0-2.5 at room temperature. After that, the solution was filtered and the samples were washed with distilled water until neutral pH was achieved (pH6.5-8.0). The samples were then dried using an oven at 80C° until constant weight was obtained. The dried sample is now known as chitin

Chitosan from chitin by alkaline hydrolysis:

The deacetylation process was conducted by soaking dried chitin prepared from demineralization in a 48% NaOH for 48 hours at room temperature. After two days, the product is known as chitosan. Chitosan was washed with tap water until neutral (pH6.5-8.0) and dried as described in deproteination and demineralization. brownish powder was obtained.

The final hybrid method

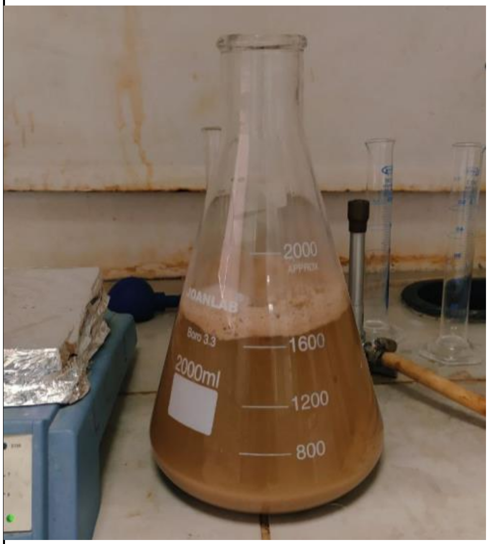
For a better chitin and chitosan extraction, choosing a method that combines the strengths of three other previous methods can be a great strategy for achieving optimal results.

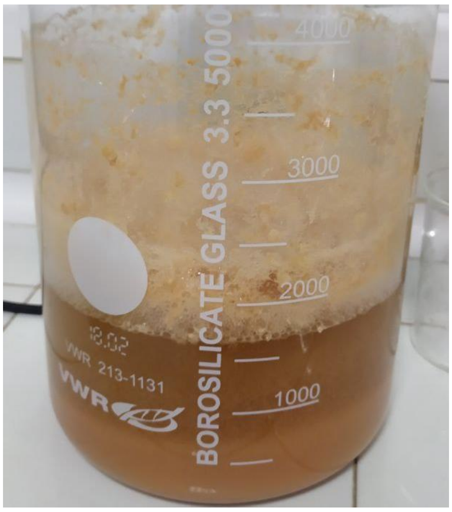
The process of developing a hybrid method may involve conducting experiments, analysing data, until we arrive at a final product that is robust, effective, and well-suited

In this particular approach, a quantity of 100 grams of shrimp shell powder was utilized to undergo a conversion process.

<u>Procedure:</u>

Table 10: Table of the final Method

Protein removal	**3h alkaline treatment** **With NaOH 5%** **Heat: 60-70 C°** **Filtered and dried in oven** **80 C°**	

Calcium carbonate removal	**1h acidic treatment With HCL 7%** **Filtered and dried in oven 80 C°**	
Colourremoval	**Acetone 24h** **Filtered and dried in oven 80 C°**	
Chitosan production	**1:30h alkaline treatment With NaOH 55% Heat: 120C° at reflux** **Filtered and dried in oven 80 C°**	

IV. Chitosan derivatives production :

Chitosan hydrochloride

chitosan is a naturally occurring biopolymer that is derived from chitin, a polymer found in the exoskeleton of crustaceans such as shrimp, crab, and lobster **(THOMAS *et al*., 2020)**. Chitosan is produced by deacetylating chitin, which removes the acetyl groups and leaves behind a positively charged polymer. This positive charge makes chitosan useful for various applications, including water treatment, drug delivery, and wound healing. **(CRINI and LICHTFOUSE, 2019).**

Chitosan hydrochloride, on the other hand, is a derivative of chitosan that has been modified with chloride ions **(THOMAS *et al*., 2020)**. This modification results in a water-soluble chitosan derivative that has improved antimicrobial properties and enhanced solubility in water. Chitosan hydrochloride is often used in cosmetic products as a natural antimicrobial agent and skin moisturizer, pharmaceutical industry as a drug delivery. **(THOMAS *et al*., 2020) (MACKAY and TAIT, 2012).**

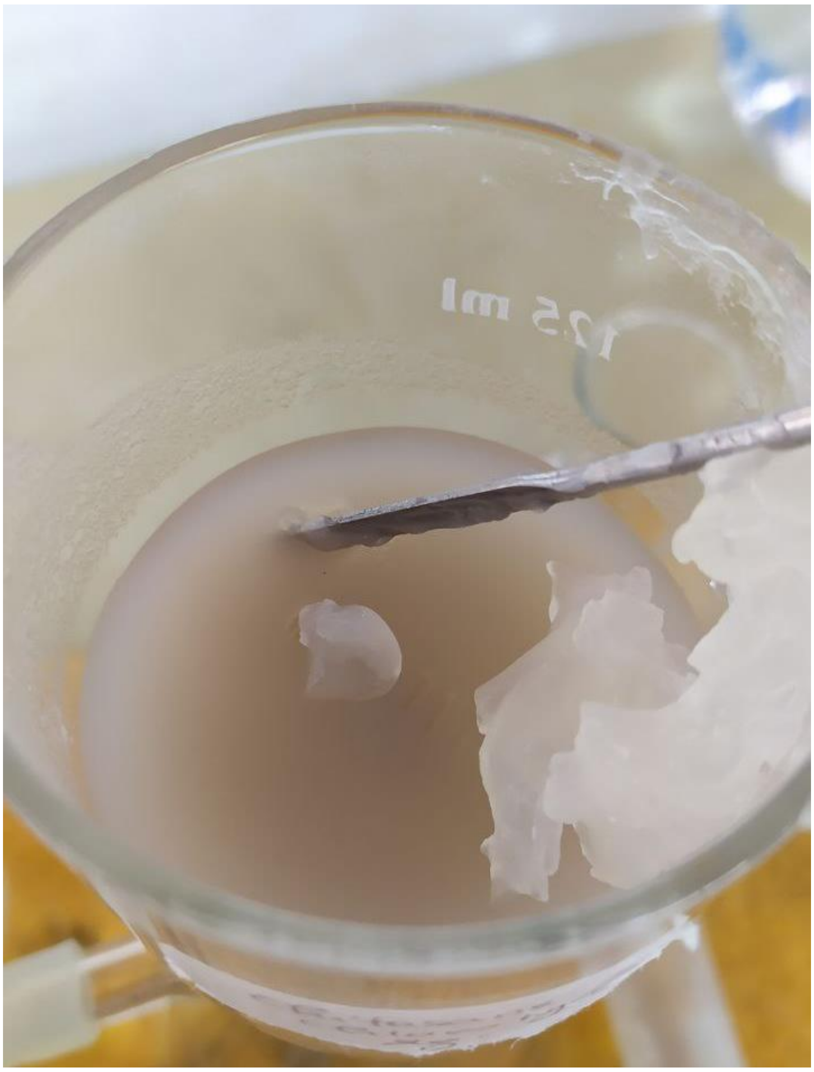


Figure 32: Chitosan Hydrochloride production

V. Physicochemical properties of chitosan

1. Determination of Total Extraction Yield

Total extraction yield is a measurement of extract mass compared with the original mass of raw material. It was calculated as the weight (g) of sample extract gained from raw material and expressed as a percentage **(AHING and WID,2016).**

The yield % was calculated by the following equation:

1

$$Yields\ (\%) = \frac{W1}{W2} \times 100$$

Where:

$W1$= Weight of dried extracted chitosan (g)

$W2$ = Weight of raw material (g)

2. Moisture content:

Moisture contents were determined by drying the sample (3 g) in a vacuum oven at 105˚C for 24h, and the decrease in the weight was measured**, (AMIN *et al*., 2022)** which corresponds to the loss of water molecules as shown in the following equation:

2

$$\text{Moist}\ \% = \frac{\text{wet sample} - \text{dried sample}}{\text{wet sample}} \times 100$$

3. Fat Binding Capacity (FBC)

FBC analysis was carried out by weighing a centrifuge tube containing 0.5 g of sample. 10 ml of vegetable oil was added and was mixed on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with shaking for 5 s at every 10 min and centrifuged at 3200 rpm for 25 min. When the supernatant was decanted, the tubes were weighed again **(NURHAYATI and TANG, 2022).**

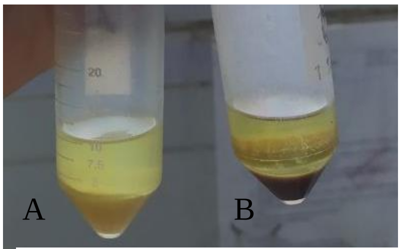


Figure 33 : FBC analysis for chitosan (A) and commercial chitosan (B)

FBC was calculated using the following equation:

3

$$FBC\ \% = \frac{fat\ bound\ (g)}{initial\ sample\ weight\ (g)} \times 100$$

4. Water Binding Capacity (WBC)

Water absorption was carried out by weighing a centrifuge tube containing 0.5 g of the sample. 10 ml of water was added and mixed on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with intermittent shaking for 5 s at every 10 min and centrifuged at 3200 rpm for 25 min. When the supernatant was decanted, the tubes were weighed again **(KUMARI *et al*., 2017).**

WBC was calculated as follows:

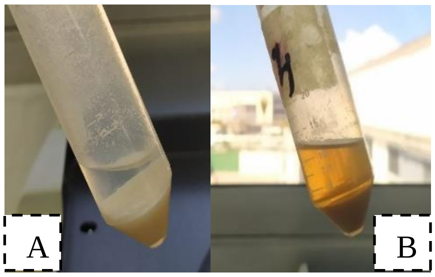


Figure 34: Wbc analysis for A"chitosan" and B"commercial chitosan"

4

$$\mathbf{WBC}\ \% = \frac{\textbf{water bound (g)}}{\textbf{initial sample weight (g)}} \times 100$$

5. Protein identification

The method used for protein identification is micro biuret method.

This method is based on the reaction between copper ions and peptide bonds in proteins, which result in the formation of a purple-colored complex, the preparation of the solution is based on pharmacopeia.

Preparation of the solution:

- Copper sulfate 5H2O:CuSO 4, 5H2O
- Double sodium and potassium tartrate: Double Na and K tartrate
- Soda:NaOH
- Potassium iodide: KI
- Distilled water

Figure 35: Biuret reagent

Procedure:

- Dissolve the copper sulphate and the tartrate in 250ml of distilled water.
- Add the 15g of NaOH.
- Add the potassium iodide and complete to 500mL with distilled water.
- Reagent to be kept in a dark place, in a polyethylene bottle carefully closed.

Materials for the identification :

- Biuret reagent
- Chitosan solution
- Chitinpowder
- Egg white solution
- Distilled water
- Test tubes

- Pipettes

Procedure :

- 1% Chitosan solution is prepared by dissolving 0.2g of chitosan powder in 20 ml of purified water
- 1% chitin solution is prepared by dissolving 0.2g of chitin powder in 20 ml of purified water
- Protein reference solution is prepared by diluting fresh egg white with same amount of purified water
- Using a pipette, add 1-5 ml of biuret reagent to each tube and mix well
- Allow the test tubes to stand for 5-10 minutes at room temperature.
- Observe any color change, The appearance of a violet color indicates the presence of protein in the sample.

6. Solubility

A dissolution experiment was conducted by introducing a predetermined quantity of chitosan powder into a 1% acetic acid solution. The solution was subjected to stirring for an extended period of time, specifically overnight, at ambient temperature. **(SULARSIH *et al*., 2012)**

The purpose of this prolonged stirring was to facilitate the dissolution process and ensure the complete integration of the chitosan powder within the acetic acid solution. This experimental procedure aimed to obtain a homogeneous chitosan solution suitable for further investigation and characterization.

7. Viscosity

The viscosity of the chitosan was determined by using a digital rotary viscosimeter at 20C° equipped with a spindle in the rotational speed and 20 revolutions per min , Chitosan solution was prepared in 1% acetic acid at 1% concentration on a dry basis**. (NURHAYATI and TANG, 2022).**

8. Molecular wight

According to **(ZARGAR *et al*., 2015)** Molecular weight can be determined by Viscometry which is a fast and uncomplicated route for the determination of the molecular weight.

The constants a and K in the Mark-Houwink equation are determined.

The intrinsic viscosity is expressed as:

5

$$\mathcal{U} = k\, M^{a}$$

Where :

$$\boldsymbol{k} = 1.81 \times 10^{-3} \text{and } \boldsymbol{a} = 0.93$$

9. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of chitin and chitosan samples were recorded using FTIR spectrophotometer (Perkin Elmer) Model: spectrum two, in the range of 400–4000 cm^{-1}.

10. Degree of deacetylation

The FTIR spectra of chitosan were obtained using an IR Instrument, with a frequency range of 4000–400 cm^{-1}.

The degree of deacetylation (DD) of the chitosan samples was calculated using the method proposed by **(SABNIS and BLOCK, 1997)**

6

$$\boldsymbol{DDA}\,\% = 97.67 - \left[26.486 \times \left(\frac{\boldsymbol{A}1655}{\boldsymbol{A}3450}\right)\right]$$

VI. Characterization of Chitosan Hydrochloride

All the tests below are conducted according to **European Pharmacopoeia 6th Edition 2008.**

Character:

Appearance	Solubility
fine white or almost white powder	fairly soluble in water practically insoluble in ethanol

Identification :

1. IR spectroscopy

IR spectra of chitosan sample is recorded using IR spectrophotometer in the range of 400–4000 cm−1, in the Physical Lab, Saidal Group, Algeria

2. S solution preparation

Dissolve 1g of chitosan hydrochloride in 100 ml of water R, maintain under vigorous mechanical stirring for 20 min

Test 01	Test 02
• **take 50 ml of S solution and complete with 250 ml of 25% ammonia solution.** ✓ **a voluminous gelatinous mass is formed.**	• **for 10 ml of S solution add 90 ml of acetone.** ✓ **a voluminous gelatinous mass is formed.**

3. Test 01 : The a- chloride reaction

Procedure:

- Use 2 ml of the prescribed solution S
- acidify with diluted nitric acid R
- add 0.4 ml of the silver nitrate solution R1
- stir and let stand, a white curd precipitate form.
- centrifuge and wash 3 times with 1 ml of water, perform this operation quickly away from bright taking into account the fact that the supernatant does not become perfectly clear.
- suspend the precipitate in 2ml of water and add 1.5 ml of ammonia
- the precipitate dissolves easily except for any large particles which dissolve slowly.

4. Ph

✓ 4-6 for S solution

5. Viscosity

The determination of viscosity was made by rotary viscometer at 20C° equipped with a spindle in the rotational speed and 20 revolutions per min.

6. Water insoluble substance

- Add 2 gr of chitosan hydrochloride to 400 ml of water, stir until the salt is completely dissolved.
- Pour the solution into a 2-litter precipitation vessel then add 200 ml of water.
- Boil gently for 2h, covering the vase to precipitate during the operation.
- Filter through sintered glass filter 40, wash the residue with water and dry to constant mass in an oven at 100-105C°.

7. Chlorides : 10-20%

Insert 0.200 g of chitosan hydrochloride into a 250 ml borosilicate flask with reflux refrigerant. Add 40 ml of a mixture of 1 volume of nitric acid R and 2 volumes of water R. Boil gently at reflux for 5 min. Cool and add 25 ml water R through the refrigerant. Add 16.0 ml of nitrate silver 0.1M and then shake vigorously. Titrate by the 0.1 M ammonium thiocyanate, in the presence of 1 ml Ferric sulphate and ammonium R2 solution, stirring vigorously approaching the turn. Perform a titration blank.

1 ml of silver nitrate 0,1 M corresponds to 3,55 mg of Cl.

8. Loss on drying : 10%

Determined in an oven at 105° on 1g of chitosan hydrochloride.

VII. Antimicrobial activity of chitosan

Chitosan, a natural biopolymer derived from chitin, has gained attention in recent years for its potential antimicrobial properties**. (AHMED and IKRAM, 2017).**

Chitosan's antimicrobial activity has been demonstrated in various applications, including wound healing, food preservation, and water treatment **(MARGUERITE and FRANCISCO, 2019)**. In wound healing, chitosan has been shown to accelerate the healing process by preventing infection and promoting tissue regeneration **(KORDESTANI, 2019).**

Several studies have reported the significant antibacterial effect of chitosan, including **(NO, 2002), (QI *et al*., 2004), (AHMED and IKRAM, 2017) (ARANAZ *et al*., 2021), (AMIN *et al*., 2022), (ABBAS and AL-SHAMMARI, 2022), (HASAN *et al*., 2022), (NGUYEN,.2021).**

In this part of the study, we will test and confirm the anti-microbial activity of chitosan produced against 3 different bacteria and a yeast Table (11), These microorganisms have relevance in various aspects of our daily life, including food safety, healthcare settings, and personal hygiene.

Table 11: Microorganisms studied, source and infections

https://www.cdc.gov/hai/organisms/pseudomonas.html

Microorganism	Source	Infections
Escherichia coli	Intestines of humans and warm-blooded animals	Foodborne outbreaks due to contaminated food (undercooked or raw meat).
Staphylococcus aureus	Skin and mucous membranes of humans	Skin infections, pneumonia, bloodstream infections, surgical site infections.
Pseudomonas aeruginosa	Soil, water, vegetation	Infections in immunocompromised individuals, particularly prevalent in hospital settings, antibiotic resistance.
Candida albicans	Gastrointestinal and genitourinary tracts, skin and mucous membranes	Oral thrush, vaginal yeast infections, invasive candidiasis in immunocompromised individuals.

1. Disk Diffusion Test

The diffusion method is one of the oldest approaches, one of the most commonly used methods in routine use, Suitable for the majority of pathogenic bacteria including slow-growing bacteria

The purpose of the disk diffusion test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds.

The pathogenic organism is grown on Mueller-Hinton agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism.

The EUCAST method is standardized, based on the principles defined in the report of the International Collaborative Study of Antimicrobial Susceptibility Testing (1972) but also on the experience of experts from around the world.

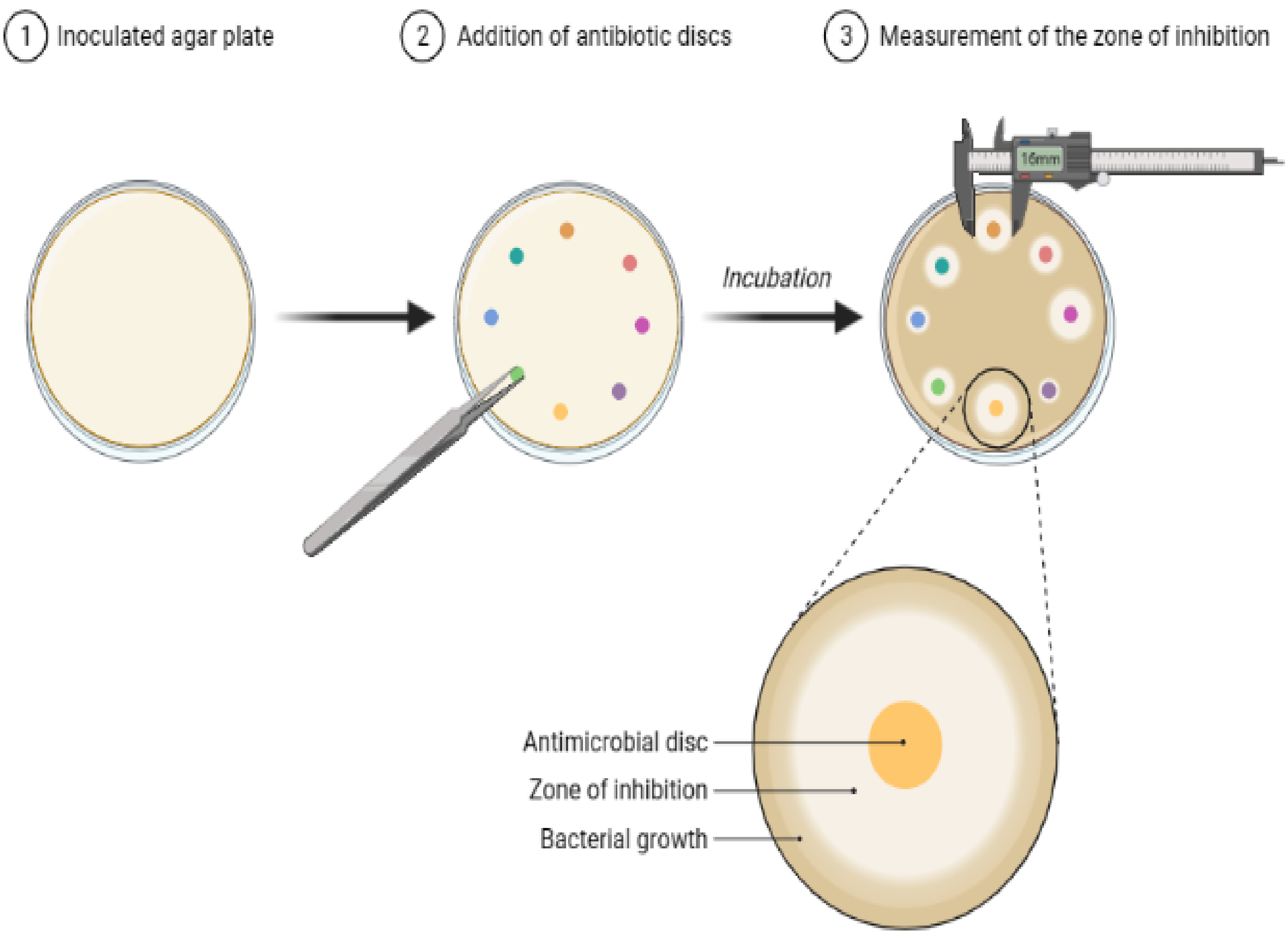


Figure 36 : Disc diffusion test

2. Mueller Hinton Agar : Preparation / Composition

<u>Preparation :</u>

- Suspend the components, dried powder, in water (38 grams in 1000 ml of purified/distilled water). The medium is boiled for a few seconds until the ingredients are completely dissolved. Autoclave to 15 lb (121°C) for 15 minutes. 15g of the powder has been used for the procedure.
- Cool to 45-50°C, mix well before pouring into sterile Petri dishes.
- Divide the medium into sterile petri dishes to obtain a thickness of 4 mm 0.5 mm (approximately 25 mL per 90 mm diameter petri dish), Allow the agar to set before moving the boxes. (Eucast 2020).

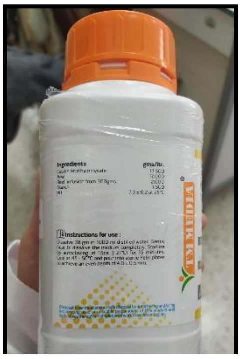


Figure 37 : Muller-Hinton culture medium

3. Microorganisms

A total of four microorganisms, comprising of two types of gram-negative bacteria, one types of gram-positive bacteria, and one fungus, were subjected to testing for the antibacterial effects of chitosan.

Table 12 : Basic features of bacterial strains used in the current study

Microbialstrain	Gram strain	ATCC
Escherichia coli	Gram -	8739
Staphylococcus aureus	Gram +	6538
Pseudomonas aeruginosa	Gram -	8626
Candida albicans	Yeast	10231

The strains were provided by Group Saidal Dar El-baida, Microbiology Department.

4. Preparation of inoculum

- Using a sterile inoculating loop or needle or a cotton swab, touch four or five isolated colonies of the organism to be tested. collect several colonies of the same morphology (if possible) to avoid selecting an atypical variant

- Suspend the organism in 2 ml of sterile saline.

- Vortex the saline tube to create a smooth suspension

- Adjust the turbidity of this suspension to a 0.5 McFarland standard by adding more organism if the suspension is too light or diluting with sterile saline if the suspension is too heavy.

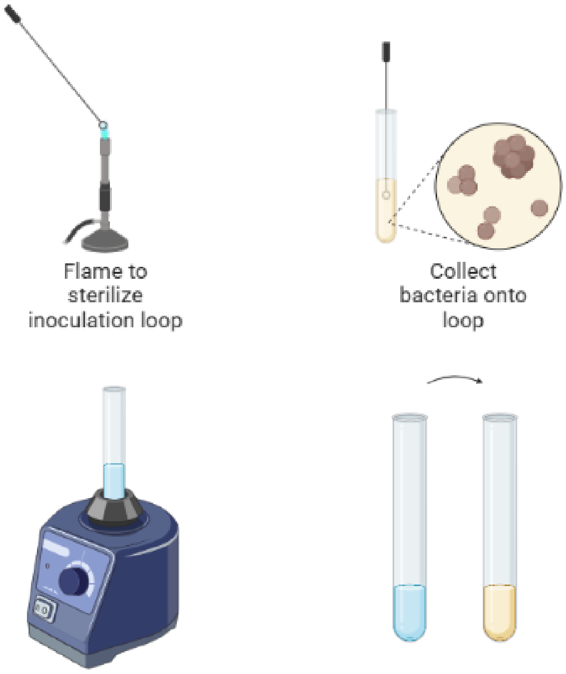


Figure 38 : Inoculum preparation

5. Chitosan Hydrochloride sample solution preparation

Chitosan hydrochloride sample was prepared at a concentration of 1% for the purpose of conducting an antimicrobial test. The preparation involved dissolving 0.5 grams of chitosan hydrochloride in 50 millilitres of a 1% appropriate solvent

The resulting solution was stirred for a duration of one hour to ensure complete dissolution. Subsequently, the solution was subjected to centrifugation at 32,000 revolutions per minute for a duration of 15 minutes. Following centrifugation, the supernatant was carefully decanted, and the gel-like residue was collected. To reduce its viscosity and facilitate absorption by the testing discs, a small quantity of proper solvent was added.

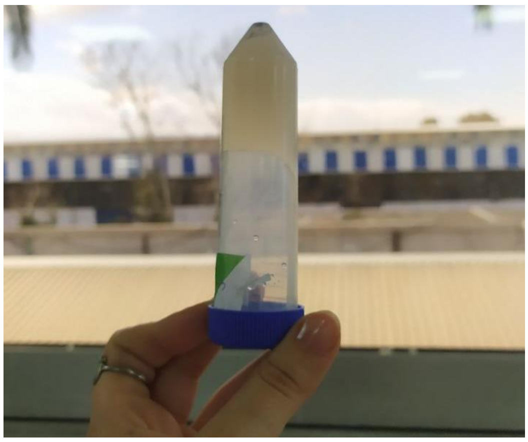


Figure 39 : Chitosan hydrochloride Gel

6. Procedure of antimicrobial testing

A. Inoculation of the MH plate

- Dip a sterile swab into the inoculum tube.

- Rotate the swab against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid. The swabshould not be dripping wet.

- Inoculate the dried surface of a MH agar plate by streaking the swab three times over the entire agar surface, rotate the plate approximately 60 degrees each time to ensure an even distribution of the inoculum

- Leaving the lid slightly ajar, allow the plate to sit at room temperature at least 3 to 5 minutes, but no more than 15 minutes, for the surface of the agar plate to dry before proceeding to the next step

B. Placement of the sterile paper disks

- The test tube must be vortexed before use to obtain a homogeneous solution

- Using sterile forceps or disk dispenser, impregnates slightly the disk in the test tube, place it on the surface of the inoculated and dried plate. Immediately press it down lightly with the instrument to ensure complete contact between the disk and the agar surface

- Once all disks are in place, replace the lid, invert the plates, and place them in a 35°C air incubator for 24h.

After 24-48 hours of incubation, the results will be measured using a capillary ruler to determine the diameter of the inhibition zone.

VIII. Chitosan-based products production

1- Wound healing – anti-inflammatory cream

Chitin and chitosan are biopolymers with excellent bioactive properties, such as biodegradability, non-toxicity, biocompatibility, haemostatic activity and antimicrobial activity **(NARAYANA *et al*., 2023)**. A wide variety of biomedical applications for chitin and chitin derivatives have been reported, including wound-healing applications. They are reported to promote rapid dermal regeneration and accelerate wound healing, **(SINGH *et al*., 2017).**

In this study, we aimed to produce a chitosan-based creams and evaluate its anti-inflammatory and wound healing properties by following a specific Saidal formulation.

Procedure:

Preparation of the oily and aqueous phases

Oily phase	
Anti-inflammatorycream	**Woundhealingcream**
Polysorbate 60	Propylene Glycol
Isopropyl myristate	Myristate
Methyl salicylate	Liquidparaffin
StearylAlcohol	GlycerolMonostearate
CetylAlcohol	Macrogol Stearate
Cetyl palmitate	CetylStearylAlcohol
Sorbitol monostearate	
BenzylAlcohol	

Figure 40: Ingredients for Cream preparation

- The aqueous phase consists of purified water.

Inversion method

- Take the two phases at the same temperature for about 10 minutes.
- Immerse them in the same 70° water bath.
- place the beaker containing the oily phase under mechanical agitation and gradually add the aqueous phase by stirring about 600 rpm
- continue to agitate until return to T° ambient.

Chitosan hydrochloride addition

Chitosan hydrochloride was dissolved in purified water, centrifuged to 3200 tr/m for 25 min, the supernatant was decanted, purified water was added to the tube and mixed well, then centrifuged again.

The supernatant was carefully poured off, and the gel component was retained.

The decision to employ chitosan hydrochloride is based on its empirically proven heightened antimicrobial properties and its compatibility with water-based skincare formulations.

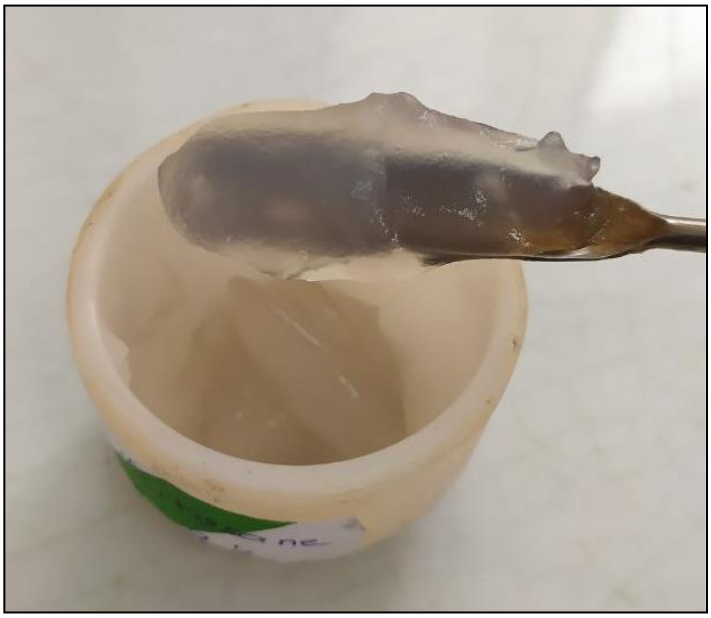


Figure 41 : Chitosan hydrochloride Gel for cream preparation

Evaluation of the cream:

Our formulated cream, intended to closely resemble market skincare creams, must undergo a series of rigorous quality controls. These encompass physicochemical evaluations such as rheological analysis and pH stability assessment, as well as sensory evaluations, including visual appearance, odor, and color assessments. These comprehensive controls ensure that our cream meets the required standards in terms of its physical properties, stability, and sensory attributes.

IX. In vivo wound healing experimentation

Wound healing is a complex biological process that involves a series of intricate cellular and molecular events aimed at restoring the structural and functional integrity of damaged tissue. One common outcome of the healing process is the formation of skin scars, which can have both cosmetic and functional implications for individuals. The development of effective strategies to enhance wound healing and minimize scar formation has been an ongoing area of research. **(KARLA *et al*, 2022).**

The utilization of chitosan-based products in vivo holds great potential for improving wound healing outcomes and reducing scar formation **(SINGH *et al*., 2017).** These products can provide a supportive scaffold for cellular attachment and migration, promote angiogenesis, modulate the inflammatory response, and enhance the deposition of extracellular matrix components **(UENO *et al*., 2001) (AZAD *et al*., 2004**). Furthermore, chitosan's biodegradability allows for its gradual degradation and integration into the healing tissue, minimizing the need for subsequent interventions. Numerous studies have reported the wound healing properties of chitosan, including studies by **ŞENEL and MCCLURE (2004), ARCHANA *et al.* (2013), CASADIDIO *et al*. (2019), ARANAZ *et al*. (2021), and NARAYANA *et al*. (2023).**

In this study, we aim to investigate the effectiveness of a novel chitosan-based product in promoting wound healing and minimizing scar formation in an in vivo model.

a) Animal selection for in vivo experiment

In this in vivo experiment, the laboratory Albio rabbit was chosen as a model. The selection of the Albio rabbit was based on several factors, including its physiological similarities to humans, ease of handling and care, availability, and previous research evidence.

The Albio rabbit *(Oryctolagus cuniculus)* is a commonly used laboratory animal in biomedical research.It shares many physiological and anatomical characteristics with humans, making it an ideal model for studying various aspects of human biology and diseases. (**GRADA *et al*., 2018).**

Figure 42: Albino rabbit

b) Material

- Albino rabbits (2 males- (1.9-2kg))
- Chitosan-based anti-inflammatorycream
- Chitosan-based wound healing cream
- Anaesthetic agent
- Sterilized surgical instruments (scissors, forceps)
- Sterile gauze
- Disposable gloves and masks
- Antiseptic solution
- Adhesive tape
- Shaving machine or razor

c) wounding Procedure

- Put on gloves, a surgical mask, Clean the surgical instruments and the work surface with an antiseptic solution, Position the rabbit in a ventral position **(FIGURE 43-A)**, with the dorsal exposed, then shave the area around the wound site, and disinfect the area with an antiseptic solution.

- Apply Local anaesthesia in the area before the procedure about 30 min to minimize pain (lidocaine 2.5 %, prilocaine 2.5%), Using a sterile scalpel blade, make a controlled incision through the superficial layer of the skin **(FIGURE 43-B)**, The wound will be allowed to bleed to ensure adequate vascularity.

- Use sterile gauze or surgical suction to control any bleeding from the wound site. Apply pressure with a sterile gauze or bandage to stop any bleeding, if necessary.

- For the cream application, a sequential application procedure was employed, wherein an initial three-day treatment period involved the administration of a chitosan based anti-inflammatory cream, followed by a subsequent application of a chitosan based wound healing cream. Apply the cream to the wound site **(FIGURE 43-C)**. Use a sterile applicator to ensure cleanliness.

- After the procedure, the rabbits were housed in a clean, comfortable environment with appropriate temperature, humidity, and ventilation, food and water as is also provided.

d) Skin irritation Test

After applying the cream, the wound site is covered with a sterile gauze patch. The first patch is removed after 5-10 min. If no serious skin reaction is observed, a second patch will be placed.

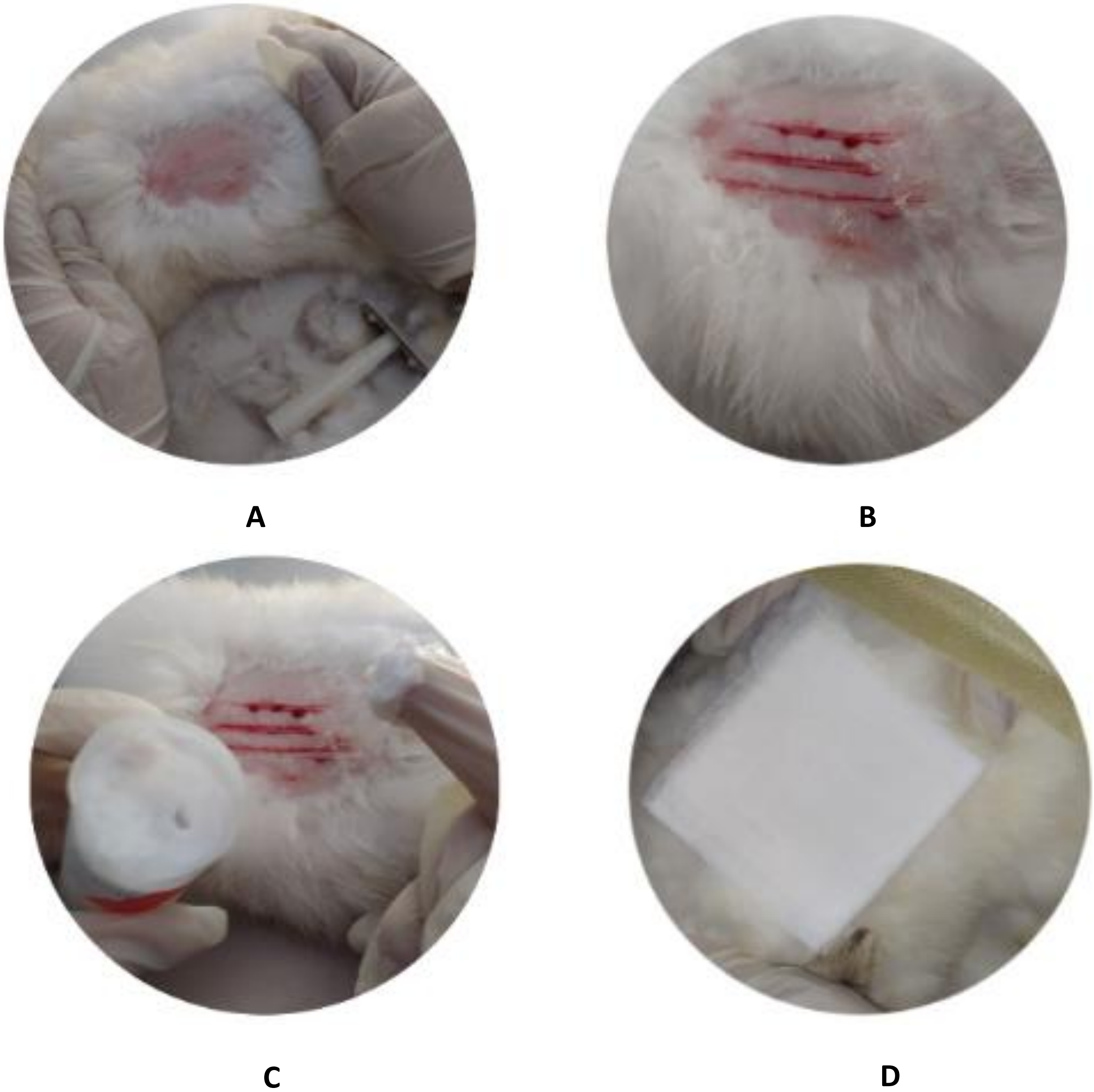


Figure 43: Wounding procedure

A) positioning the rabbit, shaving the area around the wound, and disinfecting the site.

B) make a precise incision through the outer layer of the skin.

C) Cream application

D) Covering the wound site with a sterile gauze

E) Burn-wound procedure

The objective of this second experiment was to investigate the anti-inflammatory properties of a chitosan hydrochloride-based cream. Albino rabbits were prepared for the procedure by shaving small zone of the dorsal area 24 hours prior. The site was disinfected, and an anaesthesia cream was applied 30 minutes before the initiation of the experiment.

To induce a superficial wound burn and trigger an inflammatory reaction, a sterile scalpel was heated to a temperature range of 50-60 degrees Celsius. One rabbit was treated with the chitosan-based cream, while another rabbit served as the control and was left untreated.

Figure 45: First-degree burn on rabbits skin

Figure 44 : Cream application on wound site

X. Aqueous solution for plant propagation and protection

Chitin and its derivatives are bio compounds naturally occurring in our environment, that have the potential in Agriculture with regard to controlling plant diseases.

These molecules were shown to display toxicity and inhibit fungal and bacterial growth, they have been utilized to control diseases or reduce their spread, also to collect nutrients and minerals, enhancement of root system development and enhance plant innate defences. **(EL HADRAMI *et al.*, 2010).**

Preparation of the experiment :

- For root system development the solution was prepared by dissolving 1g of chitosan hydrochloride in 99 ml of purified water.
- 10 ml of chitosan hydrochloride solution were added to 20 ml of purified water.
- The plants were put into water solutions, at room temperature and exposed to sun light**.**

The weekly progress of root development over a period of three weeks has been visually documented and presented in a table, illustrating the effects of the chitosan solution on enhancing root growth.

XI. Chitosan Films for food preservation:

Procedure :

- one beaker is filled with 2 g of chitosan and 100 ml of acetic acid.
- Under slight heating and stirring the chitosan is dissolved.
- After cooling the solution is poured through a strainer onto a plastic plate or on the backside of a rinsing bowl, The solvent is allowed to vaporize overnight.

Figure 46: 2% Chitosan solution

XII. Treatment of paper with chitosan hydrochloride:

Procedure:

In a crystallization dish 1 g of chitosan hydrochloride is dissolved in water. Some pieces of paper are dipped into the solution and then dried in hot air. With different paper samples this process is performed once, twice and three times. Ink drops are put onto untreated and treated paper samples.

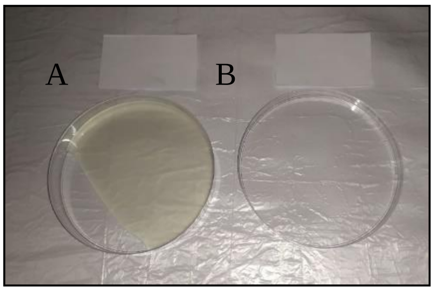


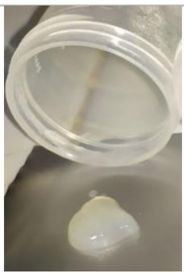
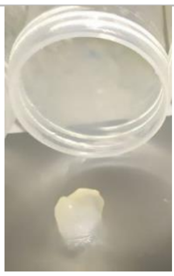
Figure 47: Treatment of paper with chitosan hydrochloride

III. Results and discussion

IV. Optimization and Evaluation of Chitin and Chitosan Extraction Methods:

The primary objective of this experiment was to evaluate and optimize three distinct methods for chitin and chitosan extraction (Table 13), the study aimed to determine the optimal conditions for chitosan extraction by considering variations in acid and base concentrations, temperature, duration, drying, and cleaning processes.

Table 13:Summary table of the 3 chitin and chitosan extraction methods

15g of the powder	Method -01	Method - 02	Method- 03
ChitinYield	20%	33%	33%
Time	1h alkaline treatment 30 min acid treatment 1h alkalinetreatment	3h alkaline treatment 24h acetone 24h acidic treatment 12h alkaline treatment	48h alkaline treatment 24h acidic treatment 48h alkaline treatment
Heat	+	+	-
Reagent %	NAOH: 2% HCL: 7% NAOH: 50%	NAOH: 5% HCL: 1% NAOH: 55%	NAOH: 2% HCL: 1% NAOH: 48 %
Colour	Slight pink	White	Brown
Chitosan amount (g)	1.5 g	3g	3g
Aspect			
Viscosity			

Various observations were made throughout the experiment, and their implications on the quality of chitosan production are summarized as follows:

- ✓ Acidic and alkaline treatments were identified as crucial factors influencing the quality of chitosan production. It was found that achieving an efficient conversion of chitin to chitosan within a relatively short time frame of 0.5-2 hours necessitates a high percentage of NaOH, typically ranging from 50-60%. This observation aligns with the findings reported by KUMARI et al. (2017), moreover an extended reaction time negatively affected the intrinsic viscosity and molecular weight of the resulting chitosan.

- ✓ Furthermore, the washing process in chitosan extraction was influenced by the use of gauze cloth, leading to the loss of chitosan powder, On the other hand, the utilization of a vacuum pump demonstrated improved results and contributed to the reduction of chitosan losses.

- ✓ Additionally, the drying process and duration played a significant role in shaping the final appearance and physicochemical properties of chitosan. The experiment demonstrated that shorter drying times in the oven yielded more desirable results. This finding suggests that minimizing the drying duration is essential for preserving the desired characteristics of chitosan and preventing potential degradation or alterations in its properties.

By combining the optimized conditions identified in the study, this hybrid approach described in the table below is anticipated to provide a superior extraction process for chitosan with improved yields and enhanced quality.

Table 14: Final hybrid chitin and chitosan extraction

Extraction Procedure	• 3h protein removal (5% NAOH-70°) • 1h calcium carbonate removal (7% HCL) • 24h bleaching (pure acetone) • 1h30 hydrolysis (55% NAOH-120°) (refluxed)
Chitin yield	• 30%
Chitosan yield	• 40%
Color and aspect of chitosan powder	• White fluffy powder

V. Chitin and Chitosan characterization results

1- Chitosan Yield

The present study has effectively generated a chitosan yield of approximately 40%, the physical characteristics of the chitin derived from the present study were brown-yellowish in appearance (a) while the resulting chitosan was white in color, odorless, and in the form of crystalline flakes (b).

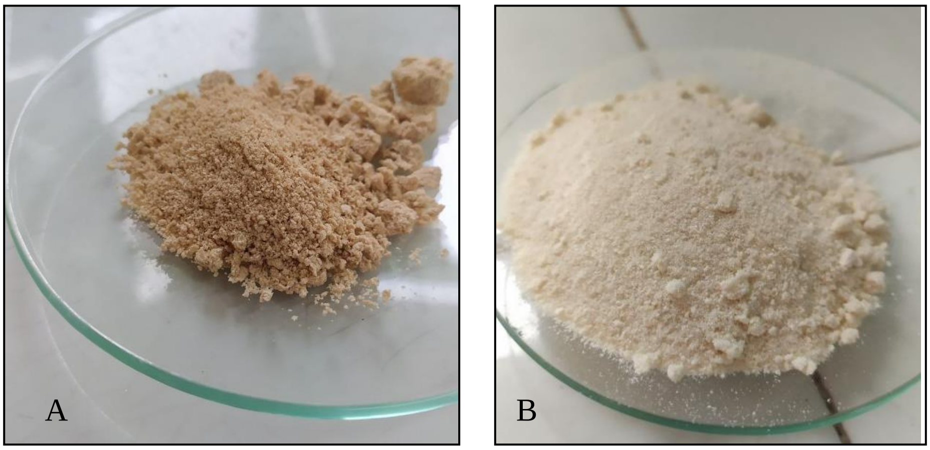


Figure 48:Chitin (A) and Chitosan (B) final aspect

The chitosan obtained in this study demonstrated improved yield and showed comparable characteristics to those documented in prior research, such as the studies conducted by **HOSNEY *et al*. (2022)** and **ALISHAHI *et al*. (2011**). The desirable white coloration of the final product serves as an indicator of the high quality of the chitosan produced, which is likely on par with the findings reported in previous investigations. This alignment is supported by the data presented in the table below:

Table 15:Chitosan yield-color findings

ChitosanYield %	Color	References
15% - 30%	Slightbrownish to white	NAZNIN, 2005
15.25%	-	ALISHAHI *et al*.,2011
15%-30%	white	HOSNEY *et al*., 2022
40%	white	PRESENT STUDY

The observed variations in yields, as investigated by **Al Hoqani*et al***., may be attributed to variances in acid and base concentrations, temperature, and deacetylation methods employed across different studies. Moreover**, AMIN *et al.* (2022)** highlighted the significance of the chitin's parent source in determining the yield (ranging from 7-40%) obtained from crustacean wastes.

2- Moisture content

The impact of moisture content on the quality of chitosan produced is a crucial factor that has been investigated in this study, The moisture content of both chitin and chitosan was quantified and is visually depicted in Figure 2.

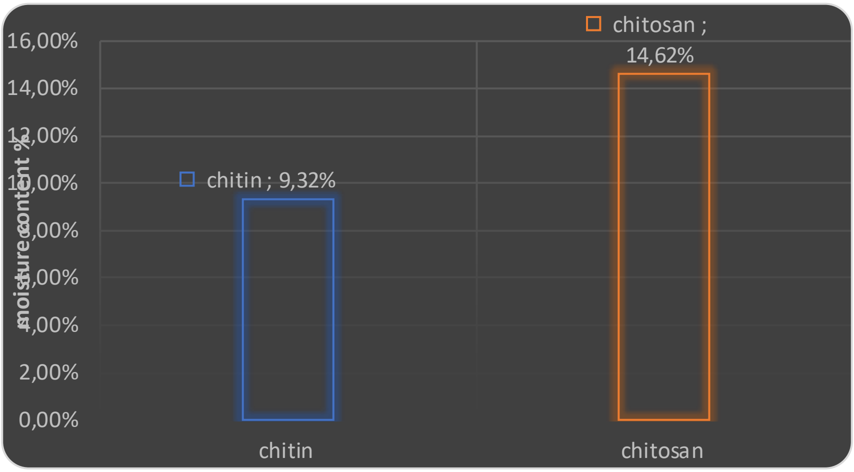


Figure 49:Moisture content of chitin and chitosan

According to (**AHING and WID.,2016**), chitin of good quality should have a moisture content of less than 5% after deproteination and demineralization processes. This is because a higher moisture content may interfere with the concentration of concentrated NaOH used in the deacetylation process, which in turn can reduce the removal rate of acetyl group from chitin and affect the degree of deacetylation of the final product. Similarly, for chitosan, it is recommended that the moisture content should be less than 10%, as compared to commercial chitosan **(HOSNEY *et al*., 2022).**

The results presented in the Figure indicate that the average moisture content of the obtained chitin was 9.32%, which is higher than the recommended level. The average percentage of moisture content in chitosan was found to be 14.62%, which is similar to the findings of (**HADDAB and KORTBi**.,**2020)**, other studies are shown in the table below:

Table 16:Moisture content findings

Moisture content %	References
9.34%	**(PUVVADA *et al*.,2012*)***
10.5%	**(KEFFIL,2020)**
14.61%	**(HADDAB and KORTBI.,2020)**
14.62%	**PRESENT STUDY 2023**

These variations in moisture content may be attributed to the different drying methods used. In the current study, oven drying was used for time-saving purposes, but previous studies have suggested that drying samples under sunlight may further reduce the moisture content in chitin and chitosan samples, leading to a lower percentage of moisture content. However, the drying time is much longer (**HOSNEY *et al*., 2022).**

3- Water and fat binding capacity

The objective of this study was to assess the water binding capacity (WBC) of chitosan, which serves as an indicator of its ability to retain water under external pressure. A higher WBC signifies that chitosan is capable of maintaining its water retention properties even when subjected to stress.

Regarding for Fat Binding Capacity (FBC) study, is to quantify the quantity of oil assimilated by chitosan. The FBC properties can be instrumental in preserving fat, taste, and consistency in diverse applications such as coatings, flavourings, and emulsions **(YADA, 2017).**

The study findings, along with relevant findings from other studies, are presented in the accompanying table.

Table 17: WBC-FBC findings

	WBC	FBC
Present study chitosan	**660%**	**494%**
Commercial chitosan tested	420%	272%
(KUMARI et al., 2017)	358%	246%
(NURHAYATI and TANG, 2022)	942% - 1374%	460%-783%
(NO, 2002) (CHO et al., 1998) **For commercial chitosan standard**	**458%-805%**	**314-535%**

The FBC and WBC results obtained from the chitosan extracted in this study exhibited superior percentages in comparison to both the commercial chitosan tested and the findings reported in the studies conducted by KUMARI et al. (2017) and NURHAYATI and TANG (2022). Moreover, when compared to the standard values mentioned by NO (2002) and CHO et al. (1998), the results further indicated the enhanced quality and performance of the chitosan produced in this study.

4- Protein identification test

The aim of this study was to identify the presence or absence of proteins in the tested samples, namely chitin, chitosan, and egg white solutions. The micro biuret method was utilized as a qualitative protein identification technique. Where the objective of this analysis was solely focused on identifying protein presence, and no attempt was made to estimate protein concentrations in the samples.

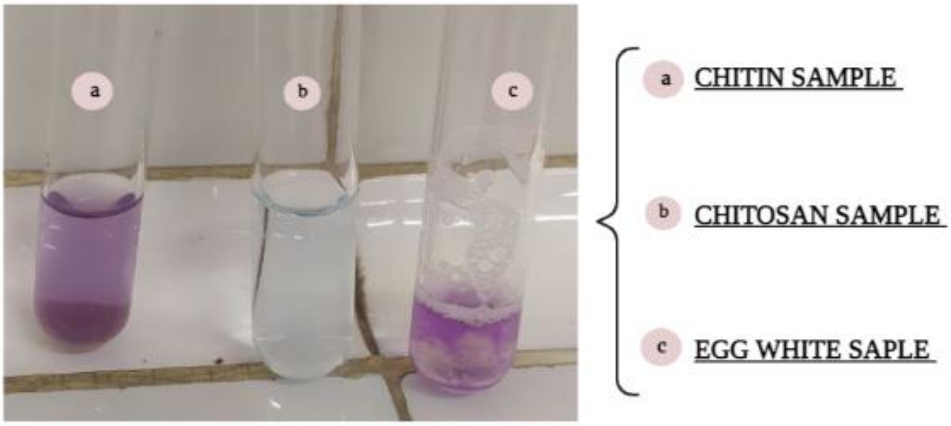


Figure 50:Protein identification test

The results of the micro biuret protein identification test indicated the presence of proteins in both the chitin and egg white solutions, as evidenced by the development of a purple color. This aligns with expectations, as egg white is known to be a rich source of proteins. However, the chitosan solution yielded a negative result with the appearance of a blue color, indicating the absence or very low levels of detectable proteins.

This supports the notion that the chitosan extraction process, involving deacetylation and thorough cleaning, effectively removed or significantly reduced the protein content. The positive result obtained for the egg white solution, which served as a reference, confirms the reliability of the micro biuret method for protein detection.

5- Solubility

The solubility of chitosan was evaluated using acetic acid as the solvent, as it is a commonly employed medium for chitosan dissolution **(SULARSIH *et al*., 2012)**, The results revealed that chitosan exhibited excellent solubility in acetic acid, forming a voluminous gel-like solution as shown in the figure below.

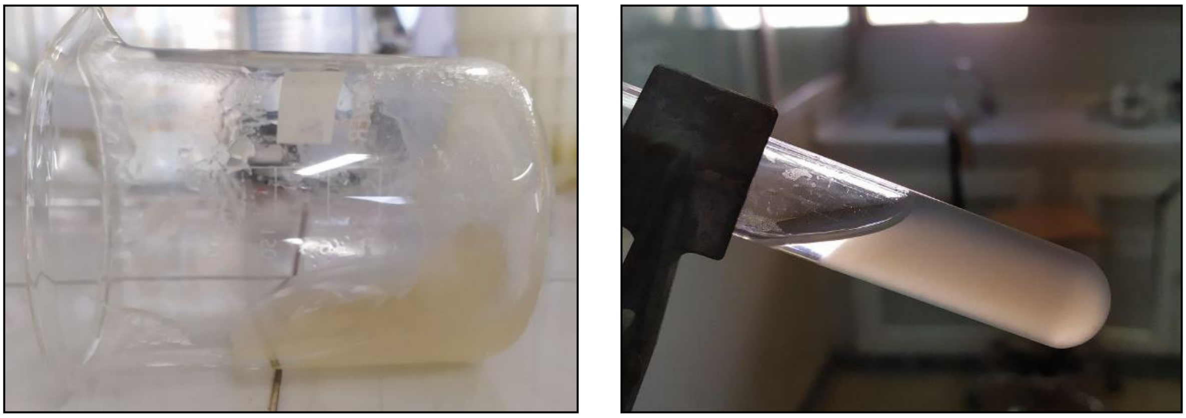


Figure 51:Chitosan solubility in acetic acid

Chitosan demonstrates poor solubility in water and both concentrated and diluted alkaline solutions. However, it exhibits solubility in weakly diluted acids, such as acetic, lactic, and citric acids. (**CRINI and LICHTFOUSE**, 2019)

The amino groups of chitosan undergo protonation starting at a pH of 3**(AHMED and IKRAM, 2017)**, resulting in the solubilization of the polymer when it acquires a positive charge , Conversely, at a pH higher than 6.5, the amino groups lose their ionization, leading to the precipitation of chitosan from the solution **(THOMAS *et al*., 2020).**

The solubility of chitosan in these acidic conditions is attributed to the protonation of its amino groups, which are essential components of its molecular structure as shown in the Figure 52 .

$$-NH_2 \quad + \quad H_2O \longleftrightarrow NH_3 \quad + \quad OH^-$$

Figure 52:Chemical reaction for amine group in chitosan with water molecule (AHING AND WID,2016)

6- Fourier-transform infrared spectroscopy (FT-IR) analysis

The FT-IR analytical technique was employed to investigate the functional groups present in chitin and chitosan. The analysis was conducted at SaidalLab, using the PerkinElmer Spectrum version 10.4.4 spectrometer, covering a frequency range of 4000-450 cm⁻¹. The obtained results are presented in the figures below:

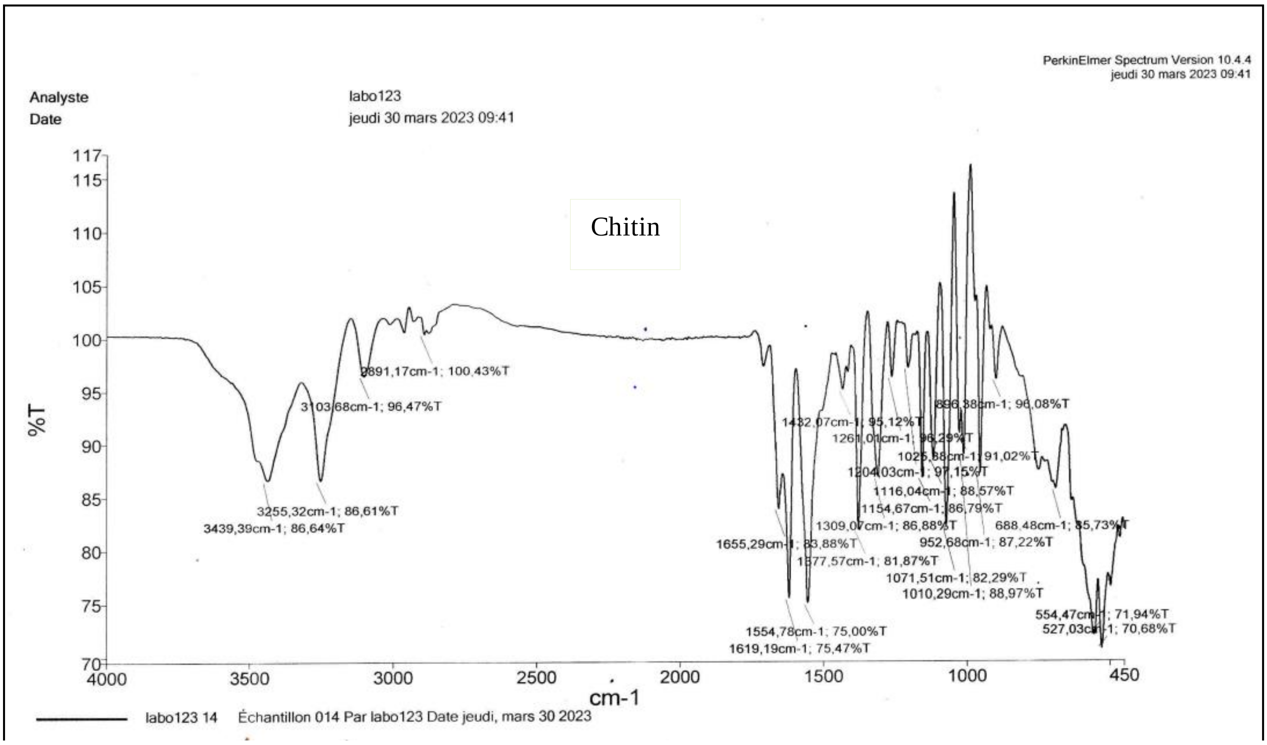


Figure 53: FT-IR analyses of Chitin sample

<u>Band characteristic in chitin sample:</u>

In the analysis of chitin using FT-IR spectroscopy, several characteristic bands were observed, indicating the presence of specific functional groups and intermolecular interactions within the molecule.

A prominent band was detected at approximately 1656 cm⁻¹, which can be attributed to the hydrogen bond formation between the amide group (C=O) of one chain and the N-H group of a neighboringintrasheet chain. This finding confirms the presence of intermolecular hydrogen bonding in β-chitin, which is a key feature of its structure. (**THOMAS *et al*., 2020).**

The stretching vibrations of N-H groups were clearly evident, with bands observed between 3264 cm⁻¹ and 3107 cm⁻¹, further supporting the identification of amine functional groups within the molecule. Additionally, the bending vibrations of two hydroxyl (OH) groups and NH groups were observed at 682 cm⁻¹, contributing to the overall chemical properties of β-chitin.

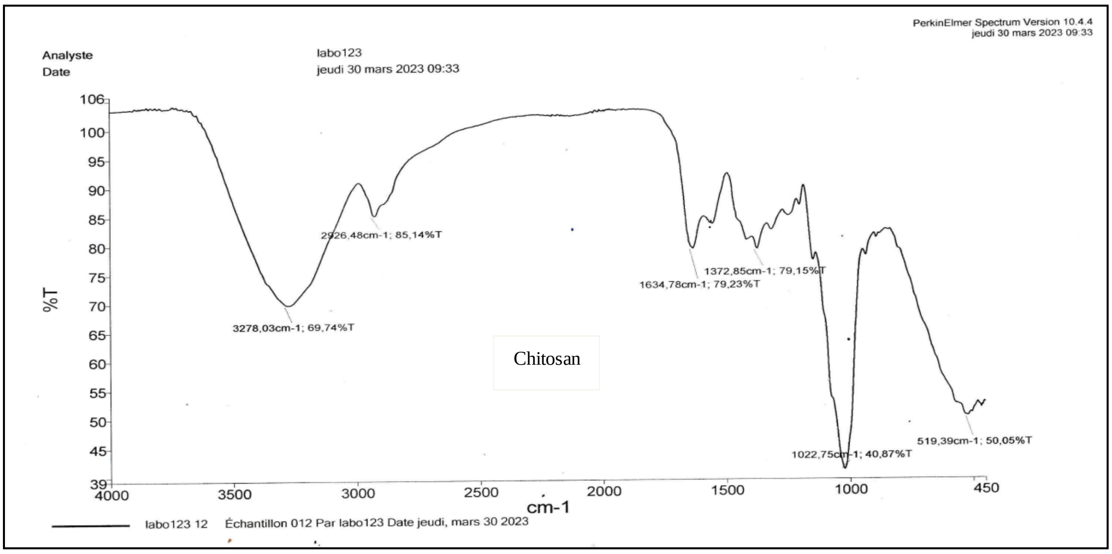


Figure 54:FT-IR analyses of chitosan sample

Characteristics IR-bands of chitosan samples:

The FT-IR spectrum of the chitosan sample reveals several characteristic IR bands associated with its chemical structure. The prominent peak observed at approximately 1650 cm^{-1} corresponds to the amide I band, indicating the presence of the C=O stretching vibration in the chitosan backbone. This band confirms the presence of acetyl groups in the chitosan molecule suggesting effective deacetylation of chitosan. The peaks observed at around 1372 cm^{-1} can be assigned to the amide II and amide III bands, respectively, further supporting the identification of amide functional groups in chitosan.

The deacetylation process involves the removal or conversion of acetyl groups to amino groups, resulting in a higher degree of deacetylation in chitosan **(MARGUERITE and FRANCISCO, 2019)**. As a consequence, the intensity or peak height of the bands at 3260 cm^{-1} and 3107 cm^{-1} decreases significantly or even disappears completely. This indicates that the N-H stretching vibrations originating from the amino groups become less prominent or negligible, suggesting a reduced presence of acetyl groups and an increase in the number of amino groups. **(THOMAS *et al*., 2020)**

7- Degree of deacetylation of chitosan

Based on the FT-IR spectrum, a proposed equation by SABNIS and BLOCK (1997), and several observations, can be inferred that the chitosan extracted from shrimp shells has a degree of deacetylation (DDA) of approximately 71%.

The DDA results obtained in this study for chitosan are superior to the findings reported by **NURHAYATI and TANG** in 2022. In their study, they conducted four experiments on chitin deacetylation, resulting in a DDA range of 55% to 67%.

In comparison, the DDA value obtained in this study, approximately 71%, indicates a higher level of deacetylation, suggesting a greater removal or conversion of acetyl groups to amino groups in the chitosan sample. These results highlight the efficacy and success of the deacetylation process in achieving a higher DDA in the chitosan extracted from shrimp shells, which can have implications for its potential applications in various fields.

8- Viscosity

The viscosity of chitosan solutions at different concentrations was measured using a digital rotary viscosimeter equipped with a spindle operating at a rotational speed of 20 revolutions per minute and a temperature of 22°C. The chitosan solutions were prepared in a 1% acetic acid solvent.

Table 18:results of dynamic viscosity of different solutions of chitosan

[chitosan]= g/ml	0.01	0.008	0.005
Dynamic viscosity mPa.s	1179.4	897.5	631.2

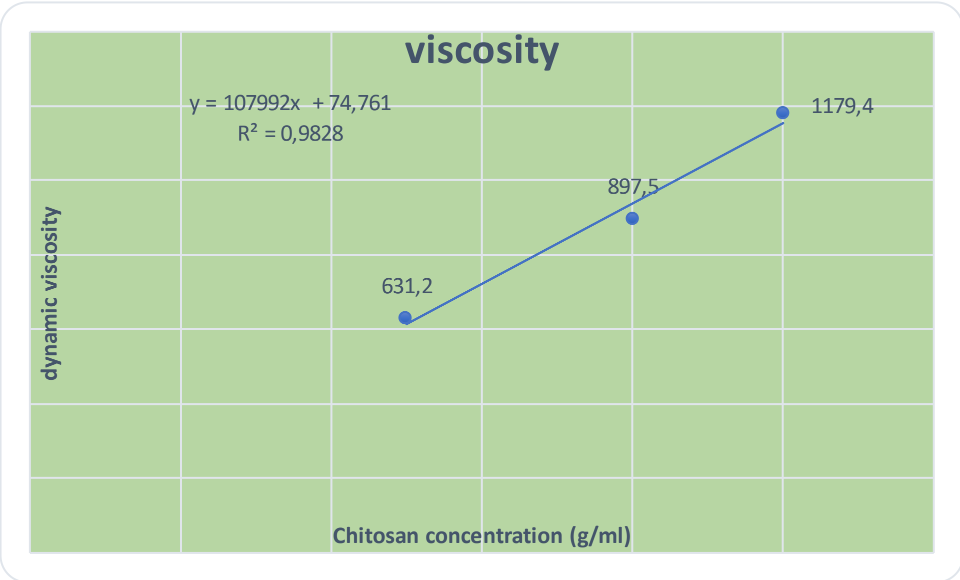


Figure 55:Determination of the intrinsic viscosity of chitosan

The vertical projection of the line on the y-axis corresponds to the intrinsic viscosity (η), The calculation method is employed to plot the graph of the relationship between intrinsic viscosity (η) and concentration (c), as expressed by the equation η = f(c).

Therefore, for x = 0, we obtain the viscosity of the produced chitosan is 74.761 ml/g.

9- Molecular weight

The method employed for calculating the molecular weight necessitates the consideration of intrinsic viscosity, utilizing the Mark-Houwink equation.

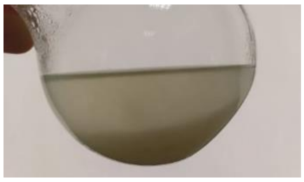
$$[M] = (\eta / K)^{\wedge}(1 / \alpha)$$

where: [M]= molecular weight, (η)= the intrinsic viscosity

The molecular weight of the produced chitosan was determined to be 130 kDa, classifying it as a medium-molecular weight chitosan. It is important to note that the standard molecular weight range for chitosan typically falls between 100 and 500 kDa (**THOMAS *et al.*, 2020**). Therefore, the obtained molecular weight value of 130 kDa is within the expected range for chitosan.

VI. Characterization of Chitosan Hydrochloride: European Pharmacopoeia 6th Edition Analysis

Table 19 : Summary of pharmacopoeia test results for chitosan hydrochloride characterization

Tests	Results	Resultpicture
Appearance	✓ white to off-white powder	
solubility	✓ solubility in water and acidic aqueous solutions	
Viscosity	✓ gel forming property	
Test 01 – acetone solution	✓ a voluminous gelatinous mass is formed.	
Test 02-25% ammonia solution	✓ a voluminous gelatinous mass is formed.	

a-Chloride reaction Test	✓ the precipitate dissolves easily except for any large particles which dissolve slowly.	
Water insoluble substance	✓ The mass of the residue is at most 10mg.	

Table 20: Physicochemical characterization of chitosan hydrochloride

Viscosity (1%)	Ph [4-6]	Loss on drying [10%]	Chlorides [10-20%]
851 mPas	6.145	30%	19%

FT-IR analysis of chitosan hydrochloride

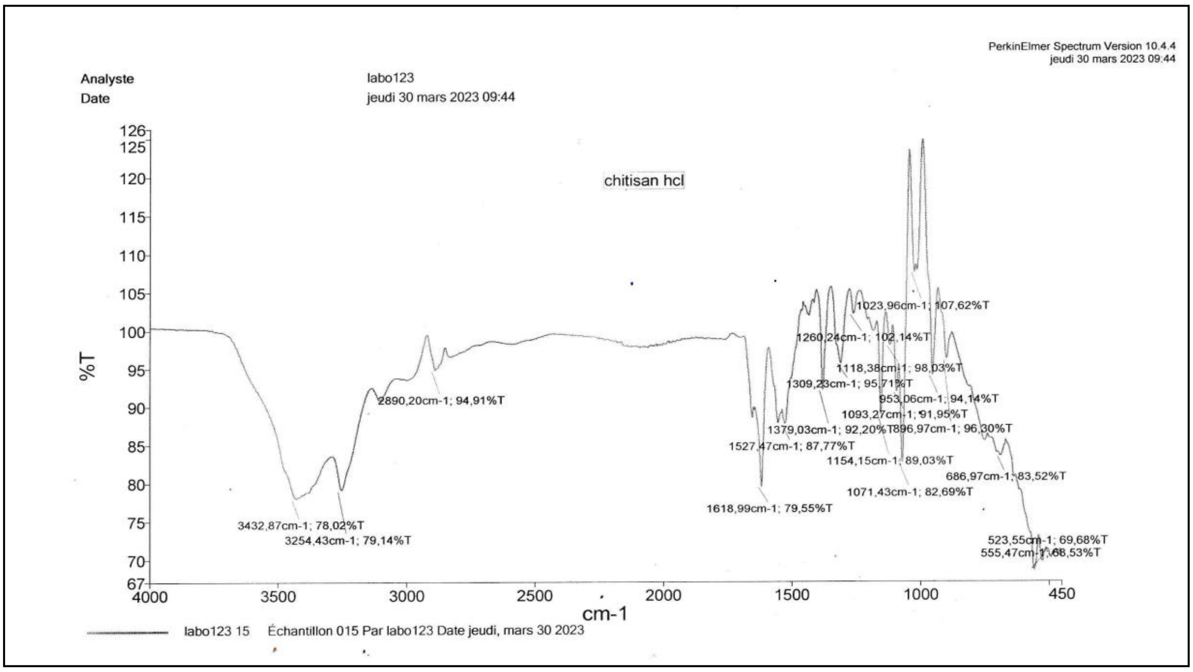


Figure 56: FT-IR analysis of Chitosan hydrochloride

Interpretation:

The prominent peak observed around 3430 cm^{-1} corresponds to the stretching vibration of hydroxyl (OH) groups, indicating the presence of primary and secondary hydroxyl groups in chitosan hydrochloride. This peak signifies the potential for hydrogen bonding and intermolecular interactions, which are crucial for its physicochemical properties and various applications.

Another important feature is the peak observed around 1400-1500 cm^{-1}, corresponding to the bending vibration of the amine groups (NH2) present in chitosan hydrochloride. This peak confirms the presence of amino functionalities and serves as an indicator of the polymer's overall composition and structure.

Furthermore, the FTIR spectrum of chitosan hydrochloride may exhibit additional peaks related to other functional groups, such as C-O stretching vibrations in the region of 1000-1200 cm^{-1}, which are associated with the glycosidic linkages in the chitosan backbone.

Characterization Results summary

The evaluation tests of chitosan hydrochloride revealed promising results regarding its suitability for pharmaceutical applications. The results indicated the consistent quality and purity of the chitosan hydrochloride samples. The pH measurements demonstrated an appropriate acidic range, facilitating its compatibility with various drug substances and formulations. Furthermore, solubility tests demonstrated its excellent solubility characteristics, which is crucial for effective drug delivery systems. The viscosity analysis revealed a suitable range for the intended pharmaceutical applications, allowing the formulation of various dosage forms such as gels and solutions.

VII. Antimicrobial activity of chitosan

The photographs depicted in **Figure 57** illustrate the antibacterial activity of chitosan hydrochloride against the tested microorganisms. The presence of inhibition zones surrounding the discs impregnated with chitosan hydrochloride suggests a significant antimicrobial potential.

The findings of this study revealed that chitosan hydrochloride displayed superior antibacterial activity against Gram-negative bacteria and fungus compared to Gram-positive bacteria.

The observed enhancement in antibacterial capabilities of chitosan hydrochloride can be attributed to its low pH, resulting in an increased number of positive charges. This finding is consistent with previous reports by **AMIN *et al*. (2022)**, who reported that the positive charges on chitosan contribute to its antibacterial activity. Furthermore, **SIKORSKI *et al*. (2022)** investigated the effects of modifying chitosan hydrochloride-based materials on microbial growth and found a complete absence of growth in both Escherichia coli and Staphylococcus aureus.

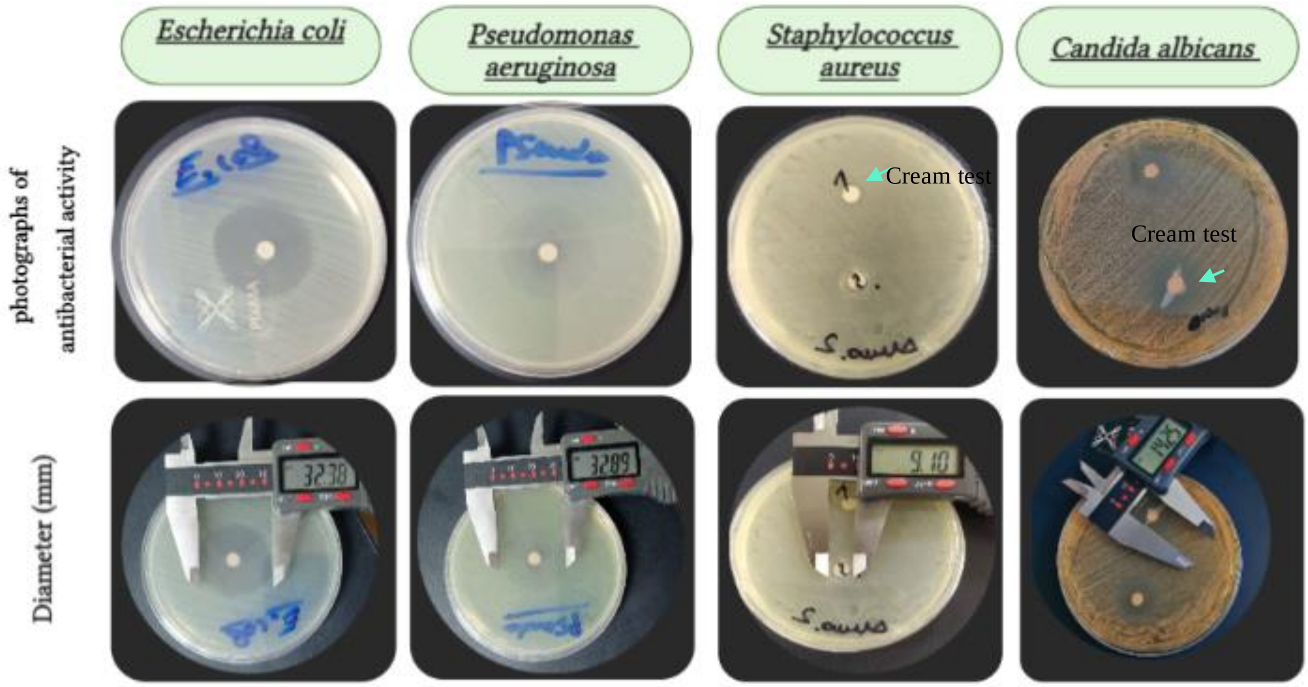


Figure 57: Antimicrobial activity of chitosan hydrochloride

However, it is essential to acknowledge that earlier studies have reported conflicting results regarding the antibacterial activity of chitosan against Gram-negative and Gram-positive bacteria. SUDATTA et al. (2020), Chung et al. (2004), and No et al. (2002) have documented instances where chitosan exhibited a higher antimicrobial efficacy against Gram-positive bacteria. These contrasting findings may stem from multiple factors, including variations in chitosan characteristics (such as molecular weight, degree of deacetylation, solvent), bacterial strains used, experimental conditions, and methodologies employed in each study.

Chitosan hydrochloride based cream evaluation tests

Based on the physicochemical tests and sensory evaluation conducted on chitosan hydrochloride-based cream, the results indicate positive findings which are discussed below:

1. Physico-chemical Tests :

- **PH**: The pH range of [5-6] is considered ideal for skincare products especially for wound healing. This pH range is close to the natural pH of the skin, which helps maintain the skin's barrier function and minimize the risk of irritation or disruption of the skin's acid mantle.
- **Viscosity**: A viscosity value of 41959 mPas suggests that the cream has a relatively high viscosity. This thick consistency is beneficial for certain applications, such as wound healing or prolonged contact with the skin. A higher viscosity can provide a more substantial barrier effect, enhancing the cream's moisturizing and protective properties.

2. Sensory Evaluation :

- **Visual Appearance**: The white visual appearance of the cream is typically associated with cleanliness and purity. The absence of any noticeable impurities or discoloration indicates good quality control during production.
- **Texture**: The cream has a heavy texture, more similar to pomade than a traditional cream. The variation in texture observed can be attributed to the inclusion of chitosan hydrochloride, which contributes to an increase in viscosity, leading to a thicker and more viscous texture. This specific texture characteristic is considered beneficial for our cream intended for wound healing purposes.
- **Odor**: The absence of any odor in the cream is generally positive. It suggests that the formulation is well-controlled and does not contain any unpleasant or irritating fragrances.

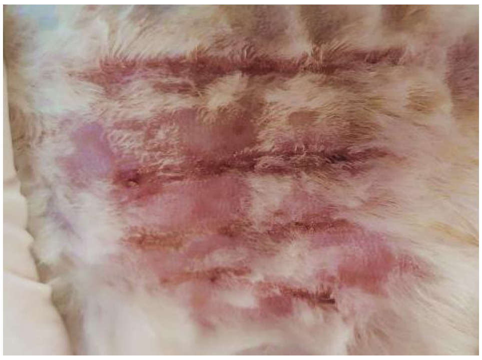
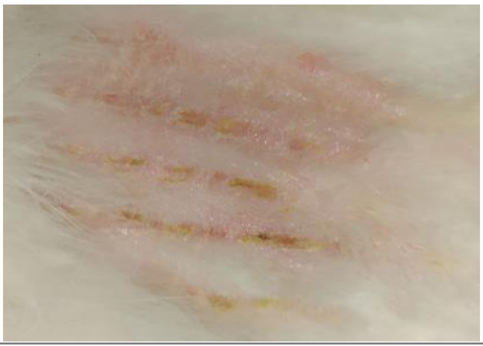
Figure 58: Cream apparence

Figure 59:Chitosan hydrochloride gel texture

VIII. In vivo Experiment Results

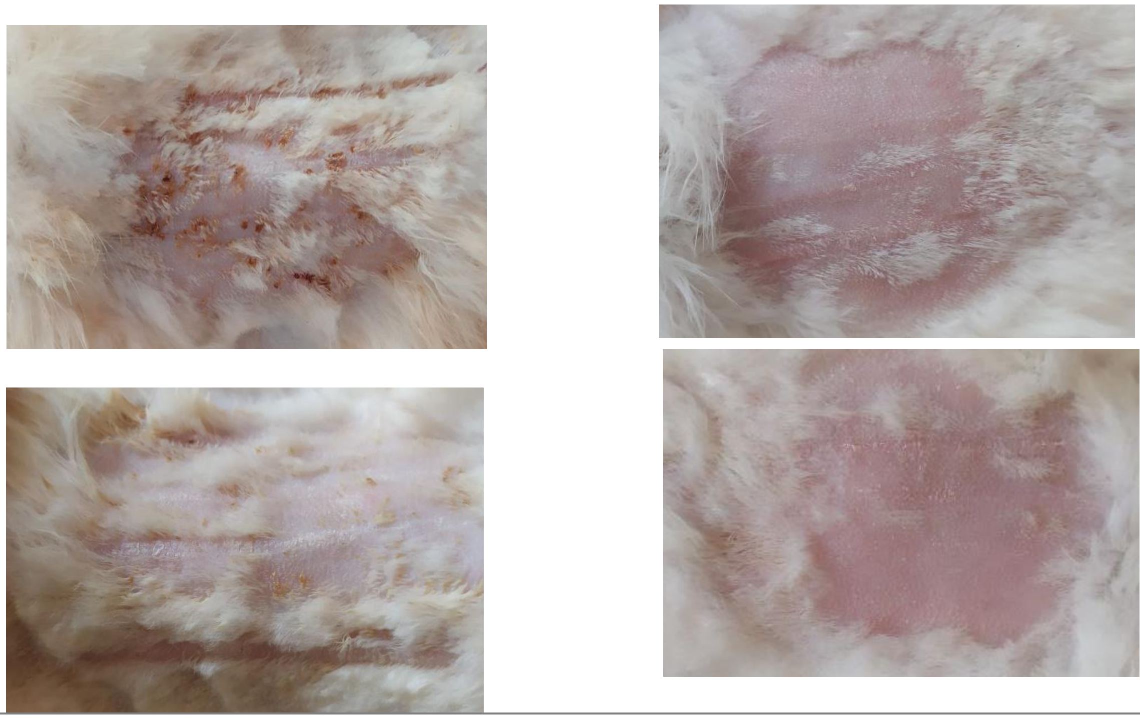
The in vivo results of the experiment on albino rabbits support the effectiveness chitosan hydrochloride-based creams. The observation table below of 15 days allowed for a detailed analysis of the healing progress and its impact on wound characteristics. The inclusion of the table provided a clear picture of the wound development over time, highlighting the positive effects of the sequential cream application on wound closure.

Table 21 :Observation table-wound healing progress over 15 days

	Non treated Rabbit (W1)	Treated Rabbit (W2)
DAY 00		
DAY 03		
Day 07		

DAY 10

DAY 15



Results discussion :

Based on the observations from the 15-day in vivo experiment on wound healing, several observations can be highlighted:

Day 3, it was observed that the treated rabbit showed no signs of redness around the wound, indicating successful management of inflammation. Additionally, the wound appeared clean, free from any signs of infection. In contrast, the untreated rabbit exhibited slight redness, suggesting a continued inflammatory response, and the wound showed no noticeable progress.

Day 7, significant progress in wound closure was observed in the treated rabbit compared to the untreated rabbit. The wound in the treated rabbit exhibited accelerated closure, indicating a more efficient healing process. Conversely, the untreated rabbit's wound showed slower progress in healing, suggesting a delay in the closure process.

Day 10, the treated rabbit demonstrated signs of mature healing, characterized by a clean wound area without any crusts. This indicated a well-established healing process, with the wound progressing towards complete closure. In contrast, the untreated rabbit's wound was still in the healing phase, with the appearance of crusts, suggesting ongoing inflammation and slower overall healing.

Day 15, both the treated and untreated rabbits exhibited complete wound healing. However, there was a notable difference in the quality of the healed skin between the two.

The treated rabbit displayed a healthier and softer skin texture compared to the untreated rabbit. This indicates that the treatment had a positive impact on the regenerative processes involved in skin remodelling and tissue regeneration. The use of the chitosan-based cream likely contributed to the formation of healthier, more robust skin, enhancing the overall outcome.

These results demonstrate the significant benefits of the chitosan-based cream in wound healing and anti-inflammatory. Its anti-inflammatory, antimicrobial, and tissue regenerative properties contribute to accelerated wound closure, improved healing outcomes, and enhanced skin appearance.

IX. Chitosan hydrochloride solution effect on root growth experiment results

- A refers to the plant soaked in purified water solution
- B refers to the plant soaked in chitosan hydrochloride solution

Table 22: Weekly root development

Week	A	B
		
02		
03		

Results and discussion :

The results indicate that the application of chitosan hydrochloride solution had a positive impact on root development in the spider plant. The treated plant (B) exhibited several advantageous characteristics compared to the plant treated with purified water (A).

Table 23:Plant observation results

Root growth	Root size	Water absorption
✓ (B) displayed longer roots compared to (A). This suggests that chitosan has the potential to stimulate root elongation and promote overall root growth	✓ The roots of (B) plant were larger in size. This indicates that chitosan may contribute to increased root biomass, which can have beneficial effects on nutrient uptake and plant health.	✓ (B) exhibited an absorbent felt, indicating improved water absorption capacity of the roots. Chitosan has been reported to have water-holding properties, which may help retain moisture in the root zone, promoting better hydration and nutrient uptake.

The observed effects of chitosan hydrochloride solution on root development in the spider plant support the notion that chitosan can be a beneficial treatment for enhancing root growth in plants. These findings align with previous research that has demonstrated the positive impact of chitosan on plant growth and development such as **(EL HADRAMI *et al.*, 2010**) and **(MARGUERITE and FRANCISCO, 2019)** studies.

X. Chitosan Films for food preservation

My initial experiment did not yield suitable results for practical use. However, the films did not meet the desired criteria for effective food preservation. The challenges I encountered were primarily related to the unavailability of specific reagents necessary for preparing the films in a suitable manner.

Despite the limitations, my experiment suggests that utilizing chitosan for food preservation holds promise due to its antibacterial potential. Chitosan is known for its antimicrobial properties, which can inhibit the growth of various microorganisms and potentially extend the shelf life of food products.

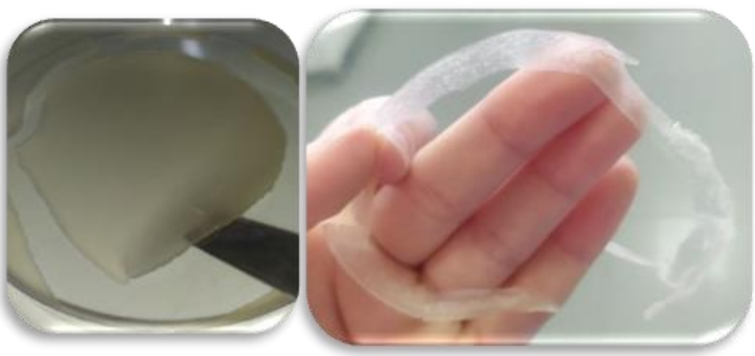


Figure 60:Chitosan films

XI. Paper treatment with chitosan hydrochloride

When comparing the paper treated with chitosan hydrochloride (A) to the untreated paper (B), several noticeable differences were observed in the Figure below:

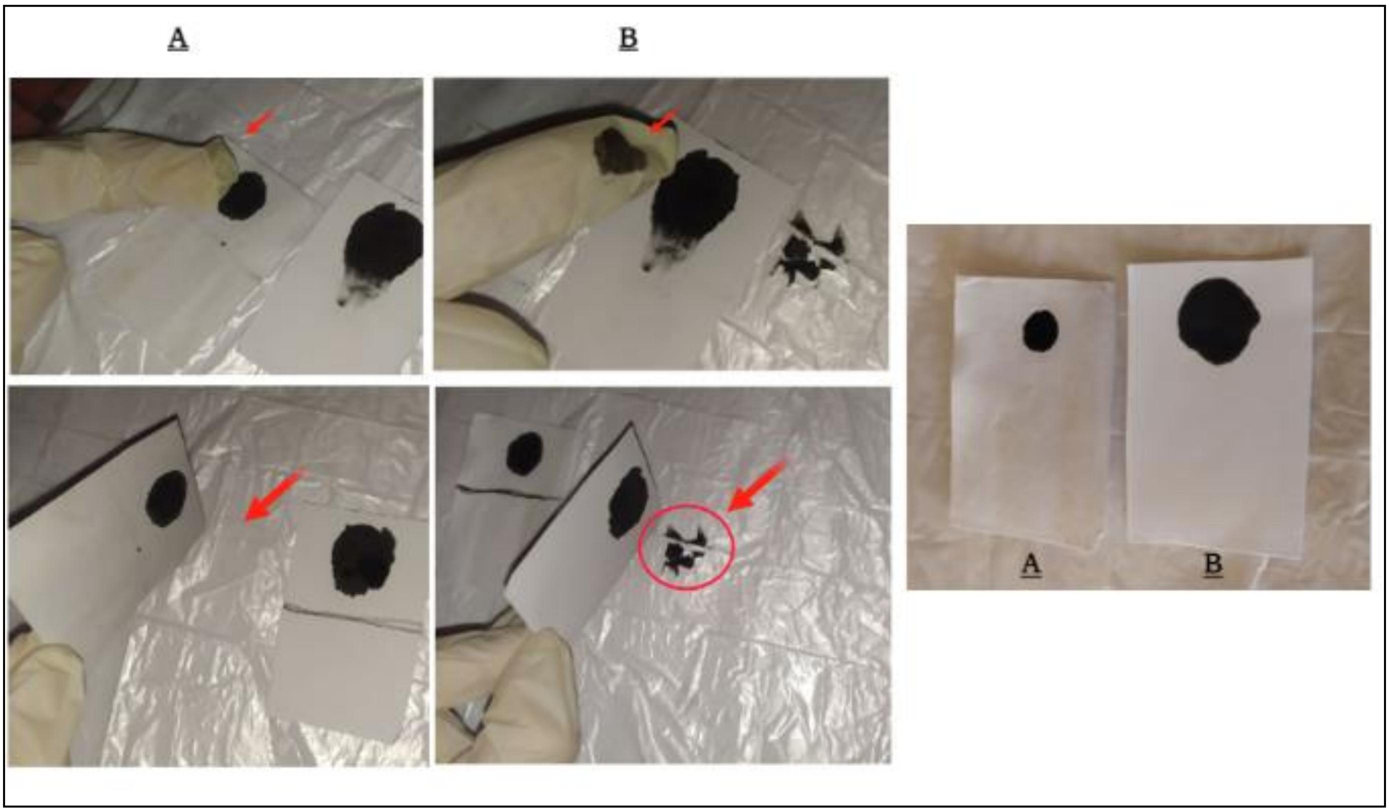


Figure 61:Treatment of paper with chitosan hydrochloride

Table 24:Observation results of Chitosan Hydrochloride paper treatment

Absorption	Strength	Retentionability
The absorption rate of ink on paper A was significantly slower compared to paper B. Ink took longer to penetrate and spread on paper A, indicating reduced ink absorbency. In contrast, paper B exhibited a faster rate of ink absorption, allowing the ink to spread more quickly on its surface.	Paper A demonstrated improved strength and durability in comparison to paper B. It exhibited higher resistance to tearing and had a sturdier texture when handled. In contrast, paper B was relatively weaker and more prone to tearing	Paper A showed remarkable ink retention capabilities. The ink applied to paper A had minimal spreading or smudging, resulting in sharper and more defined lines. In contrast, paper B exhibited more ink spreading and smudging, leading to less precise and less visually appealing writing or printing

These observations indicate that the treatment with chitosan hydrochloride positively affects the ink absorption and physical properties of the paper, leading to improved ink control and overall print quality, this confirms that chitosan hydrochloride can be suitable for paper industry.

Conclusion and perspectives

This research aimed to investigate an optimized extraction approach for chitin and chitosan from marine waste sources in Algeria, with the objectives of reducing time duration and reagent consumption while achieving higher yields and characterization of the extracted chitin and chitosan. Furthermore, the study aimed to explore the application potential of the resulting chitosan in various fields, specifically in pharmaceuticals for wound healing and anti-inflammatory purposes, as a bio stimulant for root propagation in agriculture, as films for food preservation, and as a strength enhancer in the paper industry.

From the overall results obtained, it can be concluded that:

- The chitin extraction process resulted in a chitosan yield of 71% in this study. This indicates a high efficiency in the conversion of chitin to chitosan, showcasing the effectiveness of the extraction method employed.

- The research demonstrated that a diverse range of marine shell waste sources can be utilized for chitin and chitosan extraction. This finding highlights the feasibility of using various marine waste materials, thereby expanding the potential resources available for sustainable chitosan production.

- Chitosan hydrochloride, a derivative of chitosan, exhibited superior properties compared to chitosan itself. Chitosan hydrochloride has already been approved by pharmacopeia and is widely used in the pharmaceutical industry. Its increased solubility and enhanced antibacterial activity make it a promising candidate for pharmaceutical applications.

- The antibacterial and antifungal potential of chitosan hydrochloride was investigated, and positive results were observed. Chitosan hydrochloride displayed activity against both gram-positive and gram-negative bacteria, as well as against fungal pathogens, chitosan hydrochloride exhibited greater efficacy against gram-negative bacteria and fungi, highlighting its broad-spectrum antimicrobial properties.

- The formulation of a chitosan hydrochloride-based cream for wound healing and anti-inflammatory purposes proved successful. The cream formulation met pharmaceutical standards and underwent testing in vivo on albino rabbits, demonstrating its potential for therapeutic use. This outcome supports the development of chitosan-based pharmaceutical products for wound healing and anti-inflammatory treatments.

- The experiment focusing on root enhancement using chitosan hydrochloride yielded positive results. Chitosan hydrochloride demonstrated the ability to promote root growth and enhance root propagation in agricultural applications. This finding suggests the potential of chitosan hydrochloride as a biostimulant for plant cultivation, contributing to sustainable agricultural practices.

- The production of chitosan films, although attempted, did not yield satisfactory results in terms of their effectiveness. Further research and the incorporation of specific reagents are necessary to improve the film properties and enhance their suitability for various applications, such as food preservation and packaging.

- The incorporation of chitosan hydrochloride as a strength enhancer in paper manufacturing exhibited positive results. The addition of chitosan hydrochloride contributed to improved paper quality, enhancing its tensile strength and other mechanical properties.

Overall, these results demonstrate the significant potential of chitosan derived from marine waste sources. The high chitosan yield, diverse sources of marine waste, favorable characteristics of chitosan hydrochloride, and promising outcomes in various applications underscore the value of chitosan as a versatile and sustainable biomaterial.

For the perspectives in this study, several key areas can be considered for future research:

- Optimization of Extraction Parameters: Continuing efforts to optimize the extraction parameters is crucial. Further investigations should explore variations in temperature, pH, solvent concentration, and extraction time to achieve higher yields of chitin and chitosan. Fine-tuning these parameters can lead to more efficient and cost-effective extraction methods.

- Focus on Chitosan Derivatives: In addition to chitosan, exploring the potential of chitosan derivatives is an important avenue for further research. Investigating the modification and functionalization of chitosan molecules can enhance their properties for specific applications. Chemical modifications or grafting of chitosan can improve solubility, antimicrobial activity, or biodegradability, expanding the range of applications and increasing the versatility of chitosan-based materials.

- Exploration of Other Fields: Beyond the applications studied in this research, there are several other fields that can benefit from chitosan's unique properties. Water treatment and the development of chitosan-based adsorbents for the removal of contaminants or heavy metals from water sources is an area of interest. Additionally, investigating chitosan's potential as a fat absorbent in food and pharmaceutical industries can offer novel applications and contribute to waste reduction efforts.

By addressing these perspectives in future research, the knowledge and application potential of chitin and chitosan can be further expanded, leading to advancements in extraction methods, development of chitosan derivatives, exploration of novel applications, and the utilization of chitosan in specialized fields.

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Annex

Introduction

In today's dynamic business landscape, the Business Model Canvas (BMC) serves as a crucial tool for entrepreneurs and organizations to map out their business models and drive innovation. In this section of my project explores the application of the BMC framework to my project:**The production of high-potential biomolecules, chitin and chitosan, from marine waste sources**. Moreover, the project aims not only to extract these innovative biomolecules but also to utilize the resulting purified chitosan in **the production of a diverse range of products spanning multiple fields.**

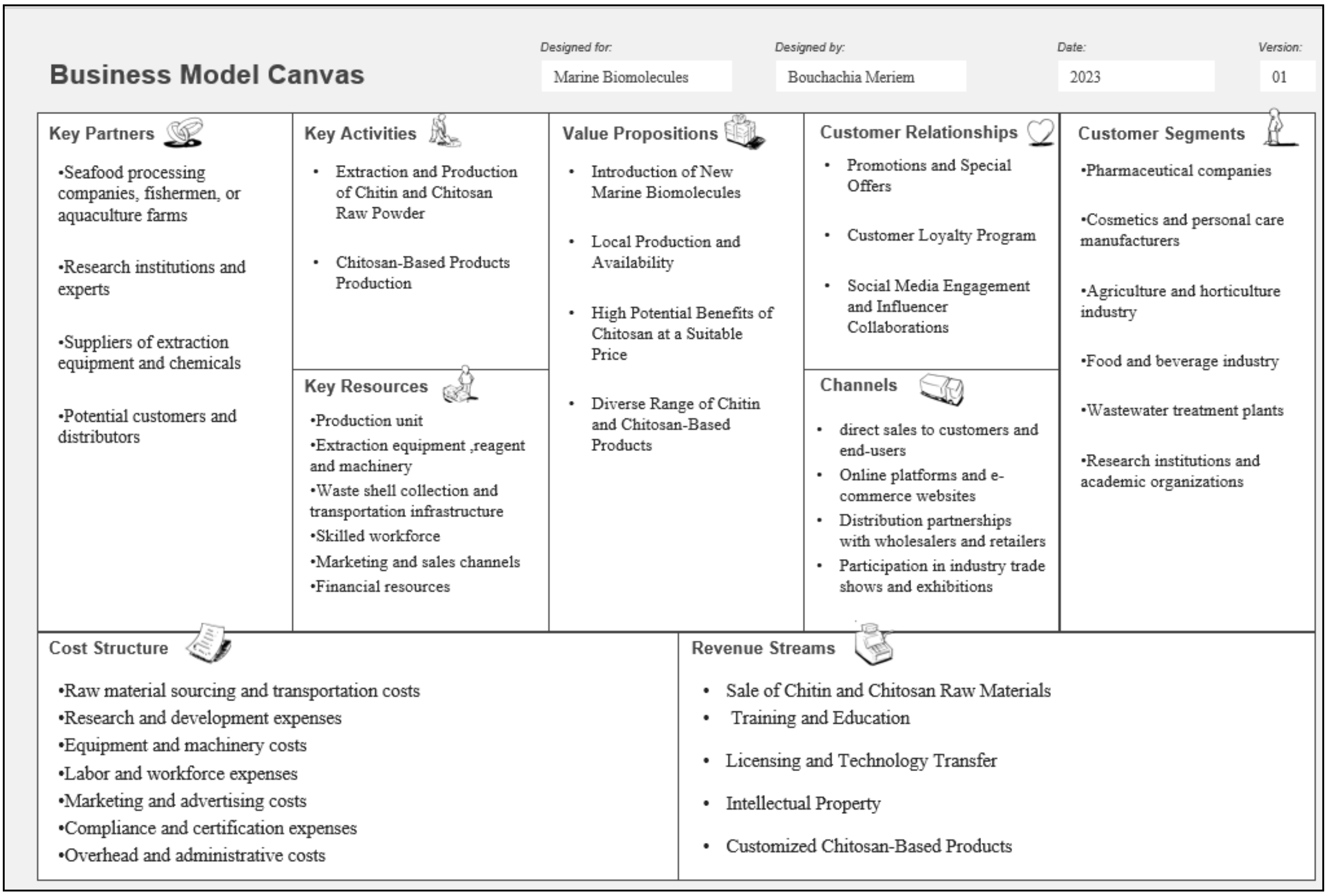
The extraction of chitin and chitosan from marine waste represents a novel and innovative approach to resource utilization. These biomolecules hold tremendous potential due to their unique properties and versatile applications across various industries. By harnessing marine waste, specifically shrimp and crab shells, this project aims to unlock the value of these underutilized resources and contribute to sustainable waste management practices in Algeria.

Moreover, the project goes beyond the mere extraction of chitin and chitosan. It seeks to leverage the resulting purified chitosan to develop a chain of value-added products across various fields. These products have the potential to impact industries such as pharmaceuticals, cosmetics, agriculture, and wastewater treatment, providing tailored solutions and opening new avenues for innovation within the Algerian market.

The BMC framework is crucial in understanding and optimizing the business model for this project. Through a comprehensive analysis of the value proposition, customer segments, channels, revenue streams, cost structure, and key partnerships, this section of my dissertation aims to uncover strategic insights and practical recommendations. By doing so, it seeks to empower entrepreneurs, investors, and policymakers with the necessary knowledge to drive the successful development and implementation of similar innovative projects within Algeria's biotechnology landscape.

This research will shed light on the significance of chitin and chitosan extraction from marine waste in Algeria, emphasizing the potential for economic growth, job creation, and environmental sustainability. By embracing innovation and capitalizing on the versatility of these biomolecules, the project aspires to shape a brighter future for the Algerian market while contributingto global advancements in biotechnology.

BMC for the project



The accompanying business model picture serves as a comprehensive visual representation of the key components and interdependencies of the business model for my chitin and chitosan extraction project. It effectively illustrates the strategic framework that forms the foundation of the project's success and outlines the various elements that contribute to its value creation and long-term sustainability. In the following sections, I will delve into each key factor in detail, providing a thorough analysis of how these components work together to drive the project's objectives and outcom

I. Key Partner

1) Seafood processing companies, fishermen, or aquaculture farms:

- These partners are crucial for sourcing shrimp and crab waste shells, which are the raw materials for chitin and chitosan extraction.
- Establishing partnerships with seafood processing companies, as they generate a significant amount of waste shells during their operations.
- Collaborating with fishermen who specialize in catching shrimp and crabs or aquaculture farms that breed these crustaceans, as they can provide a consistent supply of waste shells.
- Working closely with these partners to ensure proper collection, storage, and transportation of the waste shells to maintain their quality and freshness.
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2) Suppliers of extraction equipment and chemicals:

- To carry out the chitin and chitosan extraction process efficiently, we will need specialized equipment and chemicals.
- Identifying and make partnerships with suppliers who can provide high-quality extraction equipment such as mills, crushers, centrifuges, and filtration systems.
- Establishing relationships with chemical suppliers who can provide enzymes, acids, bases, or other chemicals required for the extraction process.
-

3) Potential customers and distributors:

- Identifying pharmaceutical companies, cosmetics and personal care manufacturers, agriculture and horticulture industry players, food and beverage companies, and wastewater treatment plants as potential customers.
- Establishing relationships with distributors who specialize in distributing chemical or agricultural products, as they can help to reach a broader customer base.
- Collaborating with these partners to understand their specific needs, provide customized solutions, and develop long-term business relationships.

Building strong and collaborative partnerships with these key stakeholders will enhance my business's sourcing capabilities, knowledge base, technical expertise, and market reach. It is important to foster open communication, mutual trust, and shared goals to ensure successful collaboration and long-term sustainability in the chitin and chitosan extraction industry.

II. Key activities

1) Extraction and Production of Chitin and Chitosan Raw Powder:

- Developing and optimizing the extraction process for chitin and chitosan from shrimp and crab waste shells.
- Determining the most suitable extraction method (e.g., chemical, enzymatic, or biological) based on factors such as yield, purity, and cost-effectiveness.
- Implementing quality control measures to ensure the extracted chitin and chitosan meet desired specifications in terms of purity, molecular weight, and deacetylation degree.

2) Chitosan-Based Products Production for Pharmaceutical and Cosmetic Fields:

- Conducting market research to identify the specific needs and demands of the pharmaceutical and cosmetic industries.
- Developing formulations and production processes for chitosan-based products, such as creams, gels, and other pharmaceutical or cosmetic formulations.
- Implementing quality control measures to ensure consistent product quality and safety.

3) Research and Development of Extraction Techniques and Chitosan Modification:

- Exploring new extraction methods or modify existing ones to improve yield, purity, or reduce the use of chemicals.
- Conducting research on chitosan modification techniques, such as chemical modifications, cross-linking, or nanoparticle formation, to enhance its properties and expand its applications.
- Evaluating the feasibility and effectiveness of modified chitosan for specific applications in the pharmaceutical and cosmetic industries.

These activities collectively support the extraction and production of high-quality chitin and chitosan raw powder, the manufacturing of chitosan-based products for pharmaceutical and cosmetic fields, and the continuous improvement and innovation in extraction techniques and chitosan modification. By focusing on these key activities, we can ensure a reliable supply of raw materials, consistent production of high-quality products, and the development of innovative solutions to meet the evolving needs of the market.

III. Key proposition

1) Extraction of High-Quality Chitin and Chitosan:

- High-quality chitin and chitosan are sought after by various industries, including pharmaceuticals, cosmetics, agriculture, and wastewater treatment, due to their unique properties and wide-ranging applications.

2) Sustainable Business Model:

- By sourcing waste shells from seafood processing companies, fishermen, or aquaculture farms, we contribute to waste reduction and promote sustainable waste management practices.

3) Diverse Range of Chitin and Chitosan-Based Products:

- My project offers a diverse range of chitin and chitosan-based products, catering to the needs of various industries.
- In the pharmaceutical industry, chitosan finds applications in drug delivery systems, wound healing products, and tissue engineering, offering improved bioavailability, controlled release, and biocompatibility.
- The cosmetic industry can benefit from chitosan's properties such as moisturization, anti-aging effects, and film-forming capabilities, enabling the production of high-quality creams, gels, and other cosmetic formulations.
- Chitosan also has applications in agriculture, where it can be used for crop protection, soil improvement, and as a bio-stimulant, promoting sustainable and environmentally friendly farming practices.

4) Customized Solutions:

- My project offers customized solutions to meet the specific needs of customers in different industries.

5) High Potential Benefits of Chitosan at a Suitable Price:

- Chitosan offers a wide range of potential benefits in various industries, including pharmaceuticals, cosmetics, agriculture, and wastewater treatment.
- The project aims to provide chitosan at a competitive and suitable price point, making it accessible to a broader customer base.

6) Local Production and Availability:

- By establishing local production of chitin and chitosan, my project ensures a reliable and consistent supply of these biomolecules.
- Local production reduces dependence on imported chitin and chitosan, minimizing supply chain risks and ensuring availability even during fluctuations in global markets.

7) Introduction of New Marine Biomolecules:

- The project introduces chitin and chitosan, derived from shrimp and crab waste shells, as novel marine biomolecules in the market.
- This novelty can attract attention and interest from industries seeking innovative and sustainable solutions, creating a competitive advantage for my project.

IV. Customer segments

1) Pharmaceutical Industry:

Pharmaceutical companies can be a significant customer segment for my project.

- Chitosan has various applications in the pharmaceutical industry, including drug delivery systems, wound healing products, tissue engineering, and biomedical coatings.
- Pharmaceutical companies seeking innovative and sustainable solutions can benefit from the high-quality chitosan raw powder and customized chitosan-based products offered by my project.
- Key customers may include pharmaceutical manufacturers, contract research organizations (CROs), and academic research institutions.

2) Cosmetic and Personal Care Industry:

The cosmetic and personal care industry presents a promising customer segment for my project.

- Chitosan is valued for its moisturizing, film-forming, and anti-aging properties, making it suitable for use in various cosmetic formulations such as creams, gels, lotions, and masks.
- Cosmetic companies, skincare product manufacturers, and beauty product suppliers can be potential customers for my chitosan-based cosmetic ingredients and finished products.
- Customized chitosan formulations tailored to specific cosmetic applications can cater to the unique needs and preferences of this customer segment.

3) Agricultural Sector:

The agricultural sector represents another customer segment for my project.

- Chitosan has demonstrated benefits in agriculture, including plant disease control, crop protection against pests, and enhancement of plant growth.
- Agricultural companies, farmers, nurseries, and agricultural research institutions can be potential customers seeking sustainable and environmentally friendly solutions for crop management and soil improvement.
- Customized chitosan formulations targeting specific agricultural applications can offer value to customers in this segment.

4) Wastewater Treatment and Environmental Remediation:

The wastewater treatment and environmental remediation industry can be an important customer segment for my project.

- Chitosan's ability to adsorb heavy metals and remove dyes from wastewater makes it a valuable material for water treatment applications.

5) Research and Development Institutions:

Research and development institutions, including universities, laboratories, and research centres, can also be potential customers.

- These institutions may require high-quality chitin and chitosan raw powder for their scientific studies, investigations, and development of novel applications.
- Collaboration with these customers can also lead to further advancements in extraction techniques, chitosan modification, and the exploration of new applications.

6) Other Industries:

Other industries, such as food and beverage, textile, paper, and biomedical sectors, may also have potential customer segments for my chitin and chitosan products.

- Chitosan's antimicrobial properties and potential use as a food preservative, textile coating, or biomedical material open up possibilities for collaboration with companies in these sectors.

V. Customer relationships

1) Personalized Customer Engagement:

- Foster personalized interactions with customers by understanding their specific needs, challenges, and goals.
- Establishing a dedicated customer support team that can promptly respond to customer queries and provide assistance when needed.

2) Continuous Education and Support:

- Educating my customers about the benefits and applications of chitin and chitosan through various channels such as webinars, workshops, product demonstrations, and educational materials.
- Provide comprehensive documentation, technical specifications, and guidelines to support customers in effectively using chitin and chitosan products.

3) Feedback Integration and Continuous Improvement:

- Actively seeking feedback from customers on their experience with my products, services, and overall engagement.

4) Promotions and Special Offers:

- Regularly running promotions and special offers to attract new customers and encourage repeat purchases.
- Promote seasonal or limited-time offers to create a sense of urgency and drive customer engagement.
- Leverage various marketing channels such as social media, email marketing, and my website to effectively communicate and promote these offers.

5) Customer Loyalty Program:

- Implement a customer loyalty program to reward and recognize the ongoing support of my customers.
- Offer loyalty cards or reward points that customers can accumulate with each purchase, which can later be redeemed for discounts, free samples, or exclusive products.

6) Social Media Engagement and Influencer Collaborations:

- Leverage social media platforms to engage with customers and create a community around my chitin and chitosan products.
- Collaborate with relevant influencers or industry experts who can endorse my products and reach a wider audience.
- Encourage user-generated content by running contests or campaigns that invite customers to share their experiences and creative uses of chitin and chitosan products.

By integrating promotions, loyalty cards, offers, and personalized recommendations into customer relationship management, we can create a sense of exclusivity, incentivize customer loyalty, and enhance the overall customer experience. These strategies can help to attract new customers, retain existing ones, and foster long-term relationships with target audience.

VI. Channels

1) Direct Sales:

- Establish my own online platform or e-commerce website where customers can directly purchase my chitin and chitosan products.
- Provide detailed product information, pricing, and an easy-to-use ordering system on my website.
- Offer secure payment options and a streamlined checkout process to ensure a smooth purchasing experience for customers.

2) Distribution Partnerships:

- Collaborate with distributors or wholesalers specializing in pharmaceuticals, cosmetics, agriculture, or other relevant industries.
- Provide necessary support, training, and marketing materials to distributors to ensure consistent product availability and representation.

3) B2B Sales:

- Develop a dedicated sales team to engage with businesses in pharmaceuticals, cosmetics, agriculture, and other relevant sectors.
- Attend industry trade shows, conferences, and exhibitions to showcase my chitin and chitosan products and establish direct connections with potential B2B customers.

4) Online Marketplaces:

- Utilizing popular online marketplaces, or specialized platforms for pharmaceuticals and cosmetics, to reach a wider customer base.
- Creating product listings and optimize them with relevant keywords, descriptions, and images to increase visibility and attract potential customers.

5) Social Media and Digital Marketing:

- Leverage social media platforms, such as Instagram, Facebook, LinkedIn, and YouTube, to build brand awareness and engage with potential customers.
- Share informative content, product updates, success stories, and application ideas to educate and attract my target audience.

By utilizing a combination of direct sales, distribution partnerships, B2B sales, online marketplaces, collaboration with research institutions, social media and digital marketing, content marketing, and influencer collaborations, i can effectively reach and engage my target audience across various channels.

VII. Potential revenue streams

1) Sale of Chitin and Chitosan Raw Materials:

- Generate revenue by selling chitin and chitosan raw materials in the form of powders or flakes to various industries, including pharmaceuticals, cosmetics, agriculture, and wastewater treatment.
- Pricing my raw materials based on factors such as purity, quality, production costs, and market demand.

2) Customized Chitosan-Based Products:

- Developing and offering customized chitosan-based products tailored to specific applications in the pharmaceutical and cosmetic industries.
- Generating revenue by selling finished products such as creams, gels, lotions, masks, and drug delivery systems.
- Pricing these products based on factors such as formulation complexity, production costs, market value, and competitive pricing.

3) Licensing and Technology Transfer:

- We have developed unique extraction techniques and innovative modifications of chitosan; we can generate revenue by licensing or transferring my technology to interested parties.

4) Training and Education:

- Providing training programs, workshops, or seminars related to chitin and chitosan extraction, modification, and application development.
- Educate and train professionals, researchers, and students in the fields of pharmaceuticals, cosmetics, agriculture, and environmental sciences.
- Generating revenue through participant fees, sponsorship, or partnership agreements with educational institutions.

5) Intellectual Property:

- Protecting intellectual property, such as patents, associated with my unique extraction techniques, modification processes, or product formulations and Generate revenue through licensing or selling the intellectual property rights to interested parties.

6) Grants and Funding:

- Seeking grants, funding, or subsidies from government programs, research institutions, or non-profit organizations supporting sustainable and innovative projects in areas such as biotechnology, waste management, or marine resources.

VIII. The cost structures

1) Raw Materials:

- The cost of acquiring shrimp and crab waste shells, which serve as the primary source for chitin and chitosan extraction.
- Costs associated with sourcing, transportation, and storage of the raw materials.

2) Equipment and Machinery:

- Investment in specialized equipment and machinery required for chitin and chitosan extraction, purification, and processing.
- Costs of maintenance, repairs, and upgrades to ensure efficient operation.

3) Labor Costs:

- Salaries, wages, and benefits for the personnel involved in the extraction process, including skilled technicians, operators, and quality control staff.
- Costs associated with training and development of the workforce.

4) Research and Development:

- Expenses related to research activities aimed at developing innovative extraction techniques, modification processes, and new applications for chitin and chitosan.
- Costs of conducting feasibility studies, experiments, and pilot projects.

5) Facilities and Utilities:

- Rent or lease payments for the facilities where chitin and chitosan extraction, processing, and production take place.
- Utilities such as electricity, water, heating, and ventilation required for the operation of the facilities.

6) Quality Control and Compliance:

- Costs associated with implementing quality control measures to ensure the purity and quality of chitin and chitosan products.
- Compliance with regulatory standards and certifications necessary for the pharmaceutical and cosmetic industries.

7) Packaging and Labeling:

- Expenses related to packaging materials, labeling, and branding of the chitin and chitosan products.
- Costs of designing and printing product labels, information inserts, and packaging materials.

8) Marketing and Sales:

- Costs associated with marketing activities, including advertising, digital marketing campaigns, and promotions.

9) Administrative and Overhead Costs:

- General administrative expenses such as office rent, utilities, insurance, legal fees, and accounting services.
- Costs of maintaining necessary licenses, permits, and intellectual property protection.

10) Distribution and Logistics:

- Expenses related to the transportation, shipping, and distribution of chitin and chitosan products to customers or distribution partners.
- Warehousing and storage costs, including inventory management and logistics operations.

Economic Estimation

The tables below provide essential information regarding the estimated costs associated with the necessary equipment for the extraction of chitin and chitosan. This table serves as a valuable resource for assessing the financial requirements of the project and helps in planning and budgeting. It outlines the approximate prices of major equipment items required for the extraction process, offering insights into the capital investment needed to establish a chitin and chitosan extraction facility.

Tableau 1: Approximate major equipment prices for chitin and chitosan extraction project

Equipment	price
Shrimp and Crab Waste Collection/Storage	
Containers/Bins	**3,000 - 10,000**
Refrigeration/FreezingUnits	**20,000 - 50,000**
Crushing and Grinding Equipment	
Crushers/Grinders	**50,000 - 150,000**
Extraction Tanks/Reactors	
Tanks/Reactors	**100,000 – 300,000**
Filtration Equipment	
Filtration Systems	**80,000 – 200,000**
Drying and Milling Equipment	
Dryers (Freeze Dryers, Spray Dryers, etc.)	**300,000 – 800,000**
Milling Machines/Grinders	**100,000 – 200,000**
Purification Equipment	
Purification Systems	**150,000-400,000**
Quality Control and Testing Equipment	
Analytical Instruments	**50,000- 400,000**
Packaging and LabelingMachinery	
Packaging Machinery	**100,000-300,000**
LabelingMachinery	**50,000-150,000**
Utilities and Infrastructure	
Plumbing and Water Supply	**Varies**
ElectricitySupply	**Varies**
Safety Equipment	
Personal Protective Equipment	**5,000-20,000**
Chemical/Waste Handling Equipment	**Varies**

Tableau 2: Approximate prices for laboratory equipment in chitin and chitosan extraction project

Equipment	Price DZD
Analytical balances	**50.000-150.000**
pH Meters	**20.000-80.000**
Centrifuges	**100.000-300.000**
Hot Plates/Stirrers	**30.000-100.000**
Autoclaves/Sterilizers	**200.000-500.000**
Spectrophotometers	**100.000-300.000**
Incubators	**150.000-400.000**
Microscopes	**80.000-400.000**
Fume Hoods	**300.000-800.000**
Lab Glassware (flasks, beakers, pipettes, etc.)	**Varies**
Lab Consumables (filters, membranes, reagents, etc.)	**Varies**
Safety Cabinets	**200.000-500.000**
LabRefrigerators/Freezers	**50.000-150.000**
Water Baths	**50.000-150.000**
UltrasonicCleaners	**30.000-100.000**

This table below presents an example estimation of the price and needs for producing one sample of a wound healing chitosan hydrochloride-based cream product in a 50 ml quantity. The estimated cost includes various components such as chitosan, other ingredients, packaging material, manufacturing costs, labeling and printing expenses, and quality control measures.

Table 25: Estimation of the price and needs for producing one sample of a chitosan-based cream

Reagent Requirements for 50 ml cream	Equipment used
Chitosan hydrochloride gel(1g)	Mixing Equipment
Emulsifiers and Stabilizers	Heating Equipment
Oils and Fats	Packaging Materials
Purified Water	Quality Control and Testing
Preservatives	

Figure 62 : Product Prototype

The production cost of the chitosan-based cream is estimated to be around **300 DZD**.

When considering the expenses associated with these components, the total production cost is expected to fall within the range of **600-800 DZD**. The packaging materials, essential for preserving and presenting the cream product, contribute to the overall expenses. Manufacturing costs, encompassing labor, equipment usage, and energy consumption, also factor into the production expenditure. Additionally, expenses related to labeling and printing, including product branding and information, contribute to the overall cost. Lastly, quality control measures, which ensure the cream's safety and compliance with regulations, are vital but contribute to the total expenses. By accounting for all these factors, the estimated range of **600-800 DZD** provides a comprehensive understanding of the total production cost of the chitosan-based cream products.

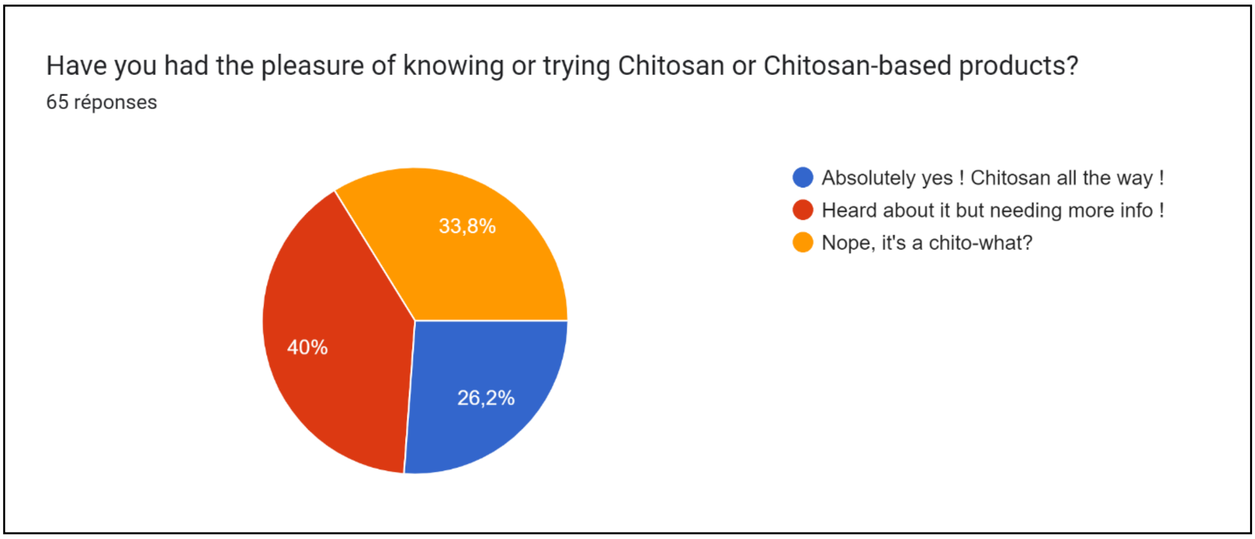
Questionnaire Results

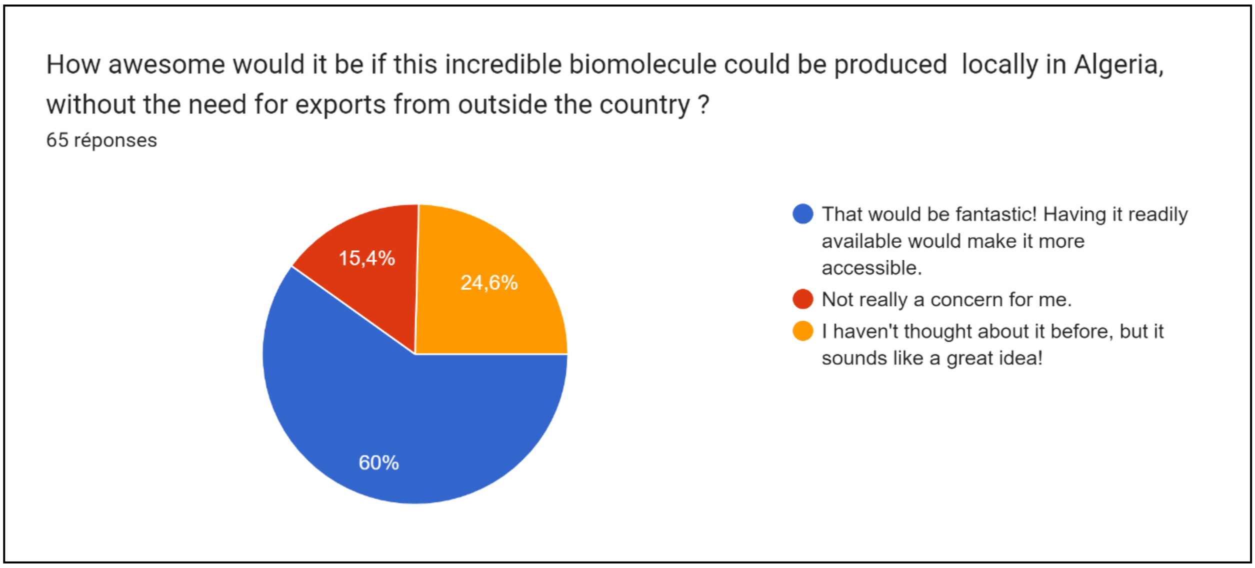
To gain valuable insights and gather feedback on the chitosan and its products, a questionnaire survey was conducted among relevant stakeholders. The purpose of this questionnaire was to assess the opinions, preferences, and perceptions of the target audience regarding various aspects of the product. By gathering responses from participants, the questionnaire aimed to provide a comprehensive understanding of the market potential, consumer preferences, and areas for improvement for the chitosan-based cream.

The questionnaire was thoughtfully answered by a diverse group of individuals, encompassing a wide range of ages (20-30) and genders.

Questionnaire URL : https://forms.gle/K6eJdZ5Lpvr8ET25A

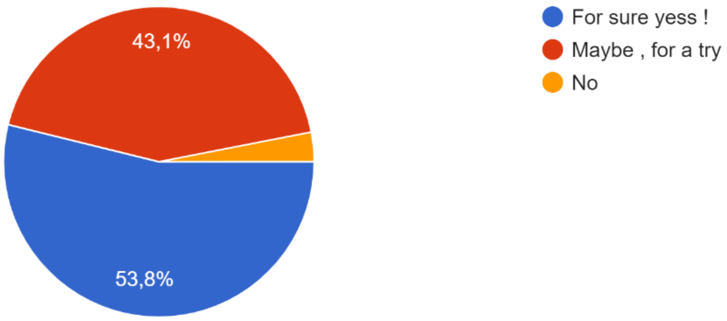
Statistic Answers





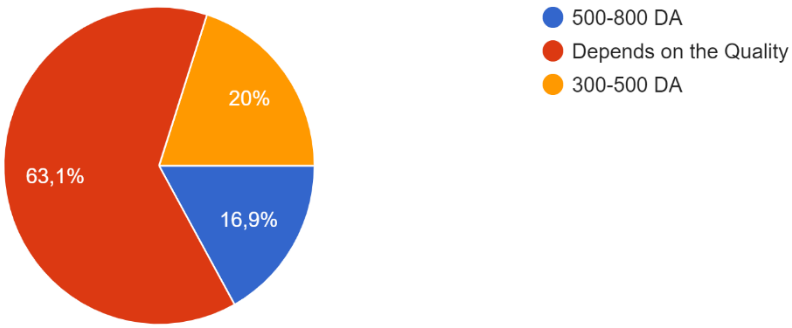
Imagine this: chitosan-based products are finally available in Algeria ! Would you be thrilled to get your hands on them?

65 réponses



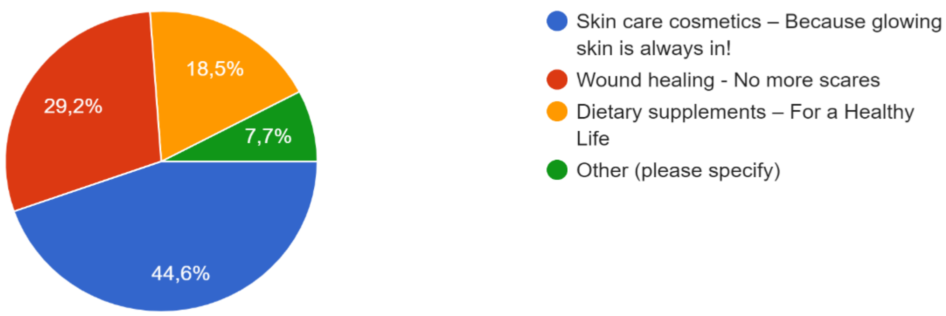
Let's talk numbers! What price range would make chitosan-based products a perfect match for your wallet?

65 réponses



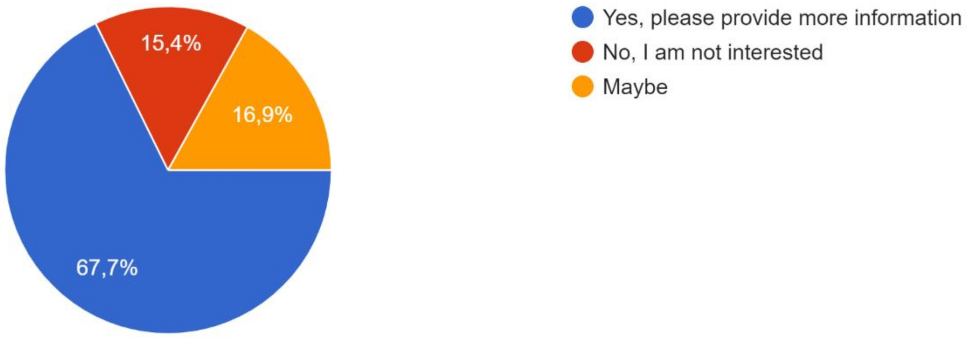
If you had the power to create a chitosan-based product, which category would you choose ?

65 réponses



Would you be interested in receiving more information about chitosan and its various applications?

65 réponses



Are you excited to experience The power of Marine Chitosan products ?

65 réponses